

MOLECULAR CHARACTERIZATION AND DETERMINATION OF
ACTIVATION REQUIREMENTS FOR A PHYTOCHELATIN SYNTHASE FROM
THALASSIOSIRA PSEUDONANA

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Phytochelatase (PC synthase) is the enzyme that synthesizes metal binding peptides, called phytochelatins (PCs), from glutathione (GSH). Found in plants and algae, PCs are produced in response to heavy metal exposure. PC production has been studied in many organisms, however PC synthase has only been isolated from a few sources. The most well-studied PC synthase was isolated from *Arabidopsis thaliana* (AtPCS1).

We studied a PC synthase from a marine diatom, *Thalassiosira pseudonana*. The gene predicted to encode this PC synthase, termed *TpPCS3*, was cloned and expressed in *E. coli* and the subsequent enzyme was purified using an immunoaffinity column. As a standard for comparison, AtPCS1 was also purified in the same manner.

The results of our enzyme assays showed that both enzymes were susceptible to oxidation following purification, however, *TpPCS3* was found to be more sensitive as it became completely oxidized during purification. Non-reducing PAGE analysis showed that oxidation of the *TpPCS3* and AtPCS1 corresponded with a change in their electrophoretic mobility. This indicates a change in the enzyme's structure which may be attributed to disulfide bond formation. Oxidation resulted in the decrease of AtPCS1 activity and the termination of *TpPCS3* activity, suggesting that preservation of reduced thiols is a necessary requirement for PC synthase activation. The optimal activation pH and temperature for *TpPCS3* and AtPCS1 were the same, however the two enzymes differed in their kinetic models and their K_M values for the substrate

Cd·GS₂. The K_M found for TpPCS3 was an order of magnitude lower than that of AtPCS1. The kinetics of TpPCS3 was best described by a ternary complex mechanism. Finally, we determined that bathocuproine, a specific copper(I) chelator, is capable of reducing the activity of purified TpPCS3 and terminating the activity of purified AtPCS1. Although determination of the metal content of AtPCS1 by ICP-MS did not prove that copper(I) was removed from the enzyme, the data still suggests that metal is necessary for activation as either a substrate or as a structural component. Thus, direct metal binding is required for either enzyme structure or activation.

BIOGRAPHICAL SKETCH

Tiffany Gupton Campolongo was born in Detroit, MI and raised in Florissant, MO. She graduated from Hazelwood Central High School in 1998. Tiffany attended Florida Agricultural and Mechanical University for her undergraduate studies, where she earned a Bachelor of Science degree in Biological and Environmental Systems Engineering in 2002. She then attended Cornell University where she earned a Master of Science degree in Biological and Environmental Engineering in 2006. Following this, Tiffany continued her Master's thesis research at Cornell en route to earning a Doctor of Philosophy degree in Biological and Environmental Engineering. In 2009 Tiffany married her best friend, Michael, and they began a life of science together. "We winnin' over here!"

To Mikey, Mom and Dad

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CHAPTER ONE

BACKGROUND

1.1 METALS IN CELLS

Heavy metals are natural components of the earth's crust. Trace amounts of essential metals, such as copper, iron, and zinc, occur naturally in the environment and are essential for life at the cellular level. However, these metals in excess can be detrimental. Redox reactive transition metals, for example, can donate or accept electrons, causing oxidative stress [1]. In addition, non-essential heavy metals, such as cadmium, arsenic, and lead, are released into the environment by industrial and agricultural processes [2]. Low concentrations of these heavy metals can be as damaging to cells as excess essential metals by inactivating proteins and peptides via thiol-capping [3].

1.2 PHYTOCHELATINS

To cope with metal contamination, plants have evolved various methods for metal detoxification. One method is the synthesis of chelators, which are high affinity metal-binding peptides or proteins. Metals bound to chelators are less reactive, thus decreasing the potential for toxicity. Phytochelatins (PCs) are one type of chelator that are synthesized in the presence of heavy metals and excess essential metals. They have been found in all plant species, most eukaryotic algae, some yeast and the nematode, *Caenorhabditis elegans* [4]. The PC amino acid sequence consists of γ -glutamylcysteine (γ -Glu-Cys) repeats that are capped at the C-terminal end with glycine. The structure of PCs is described as $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, in which the chain length n can vary from 2 to 11 [5, 6]. In addition, there are four other main classes of PCs: homo-PCs, hydroxymethyl-PCs, iso-PCs and des-Gly PCs which are capped at

the C-terminal end by β -alanine, serine, glutamic acid, or no amino acid, respectively [7].

PCs are synthesized in the cytosol of plants in response to excess essential or toxic heavy metals. A variety of metals, including cadmium, lead, zinc, silver, nickel, and copper, are capable of PC induction in plants. Of these, cadmium is the strongest inducer of PC synthesis [8] and is the model metal typically used in *in vivo* and *in vitro* assays. PCs bind tightly to heavy metals forming PC-heavy metal complexes that can be transported to the vacuoles for storage. In the vacuoles, PC-heavy metal complexes are modified by the addition of reduced sulfur. This modification allows for an increase in the number of heavy metals that can bind to the complex [9].

1.3 PHYTOCHELATIN SYNTHASE

1.3.1 Dipeptidyl transpeptidase reactions of phytochelatin synthase

PCs are synthesized by the enzyme phytochelatin synthase (PC synthase) from glutathione (GSH) which has the amino acid structure γ -Glu-Cys-Gly and is the major antioxidant inside cells. PC synthase is a dipeptidyl transpeptidase that cleaves the terminal glycine off of a GSH peptide and transfers the remaining γ -Glu-Cys peptide to another GSH peptide or PC_n chain to form PC_2 or PC_{n+1} , respectively. This was first hypothesized by Grill et al. [10]. The authors found that when PC synthase partially purified from *Silene cucubalus* cell extract was assayed in substrate buffer containing GSH and PCs with chain lengths $n = 2, 4$ or 5 , the result was the formation of PC_{n+1} , concomitant with the release of glycine. The dipeptidyl transpeptidase nature of PC synthases was unequivocally proven by Vatamaniuk et al. [11]. These researchers assayed a PC synthase cloned from *Arabidopsis thaliana*, (AtPCS1) in substrate buffer consisting of GSH containing radiolabeled glycine or radiolabeled cysteine and unlabeled PC_2 . The results of the experiments showed that upon the

formation of PC₃, radiolabeled glycine was released from GSH. In addition, 33% of the thiols in the new PC₃ peptide were radiolabeled, confirming that AtPCS1 and (by implication) all PC synthases are dipeptidyl transpeptidases.

1.3.2 Phytochelatase synthase domains

PC synthases have two domains: a highly conserved N-terminal domain and a divergent, cysteine rich C-terminal domain. The defining characteristic of the N-terminal domain is the presence of three positionally conserved amino acids: cysteine, histidine, and aspartic acid as shown in an alignment of canonical PC synthases (Figure 1) [12, 13]. Site directed mutagenesis of these amino acids in purified AtPCS1 resulted in the termination of PC synthesis, providing evidence that these three amino acids are required for enzyme catalysis [11, 14]. In addition to the catalytic cysteine, the N-terminal domains of PC synthases also contain four other positionally conserved cysteines (Figure 1). The function of these cysteines (if any) has not been determined due to conflicting results reported following their site directed mutagenesis in different PC synthases. The individual or collective substitution of the N-terminal cysteines in AtPCS1 resulted in no change in the enzyme's capability to produce PCs or rescue cadmium sensitive yeast mutants [14, 15]. In contrast, the substitution of these cysteines in a PC synthase from *S. pombe* (SpPCS) resulted in complete or partial loss of the enzyme's activity and ability to rescue cadmium sensitive yeast mutants. In this same study it was shown that the N-terminal conserved cysteines in SpPCS and a PC synthase from *Triticum aestivum* (TaPCS) participate in cadmium binding motifs, a role which may be important for activation of the enzyme [16].

TaPCS1	-----MEVASLYRRVLPSPPAVEFASAEKRLFAEALQGGTMEGFFNLISYFQTQSEPAFCGLA	59
ApPCS1	-----MAMASLYRRSLPSPPAIDFFSAEKLIFNEALQKGTMEGFFRLISYFQTQSEPAFCGLA	59
CePCS	-----MSVTAKNFYRRPLP-ETCIEFSSSELCKLFTTEALVFGSANIYFKLASQFRTQDEPAFCGLS	60
SpPCS	MNIVKRAVPELLRGMTNATPNIGLIKKNVVSFEAVGQLKKSFKRQLP-KQCLAFDSSLCKDVELRALQEGRMENYFSLAQQMVTQNEPAFCGLG	94
TaPCS1	SLSVVLNALAIDPGRPWKGPWRWFDESMLIDCEPLHKVKAEGITFGKVVCLAHAGARVQSFRAQDQTT--IHDFAHLTRCASSQDCHLISSYHR	152
ApPCS1	SLSVVLNALSIDPGRKWKGPRWRWFDESMLIDCEPLEVVKKEGISFGKVVCLAHAGSGAKVEAFRTSQST--IDDFRKFVVKCTSENCHMISTYHR	152
CePCS	TLVMVLNALEVDPEKVVWKAAPWRFYHESMLIDCCVPLENIRKSGINLQQFSQAKCNRLKSTVSQDNPDLKKFRTSLVNSVRSDQDLVASYDR	155
SpPCS	TLCMILNSLKVDPGRLWKGSRWRYDQYMLIDCCRSLSDSIEKDGVTLEEFSCLANCNGLRITITKCVKDV--FDEFKRDVISCTIENKIMAI SFCR	187
TaPCS1	SPFKQTGTGHFSPIGGYHAEKDMALIIDVARFKYPPHWPVLTLLWDAMNTTDEATGLLRGFMVSRSSAPSLLYTVSCGHGSKWSMAKYCVEDV	247
ApPCS1	GWFKQTGTGHFSPIGGYNAERDMALIIDVARFKYPPHWPVLTLLWEAMDSIDQSTGKRRGFEMLISRPHREPGLLYTLSCKDESWIEIAKYLKEDV	247
CePCS	SVLGQTGSGHFSPLAAVHEDSDQVLIMDVARFKYPPHWPVKLETQLKALCSVDVTTKLPRLGVELLKKG-----	224
SpPCS	KVLGQTGDGHFSPVGGFSESDNKILIIDVARFKYPCYVWDLKLMYESMFPIDKASGQPRGYVLE-----	252
TaPCS1	PNLLKDESLDNVTTLTLLSRVLESLPANAGDLIKCVIEVRRKEEGESSLSKEEKERFLFKEKVLQQIRDITDLFRVVHVELQYPKGLCGSCSSSDEDS	342
ApPCS1	PRLVSSQHVDSEKIIISVVFKSLPSNFNQFIRWVAEIRITEDSNQNLSAEEKSRLLKQLVLEKHETELFKHINKFLSTVG-----YEDS	333
CePCS	-----TRPLIMYGLKAYVINDSDFATSVISWNQFLCDPLEDEEFQCCRKFG-----	275
SpPCS	-----PMHIPLGVLTVGLNKYSWRNVSKHILQQAATVKNADNLAELLSINQ-----	299
TaPCS1	LAEIAATVCCQGAFLSGNLVSRDGFCCRETCTIKCIEANGDGLKTVISGTVVSKGNEQAVDLLPTSSSKTSLCNSNLKSKIWKYPSSTDVLTVL	437
ApPCS1	LYYAAAKACCCQGAELSGSPSKE--FCCRETCTVKCIKGPDDSEGTVTVGVVVRDGNQKVDLLVPSTQTECEG-----PEATYPAGNDVFTAL	420
CePCS	QCFAPHAMCCTQKTFDADQ-----KNSCTECSTDQNEACKMICS-----EIRRTRFAEVFSSS	328
SpPCS	--SSIPLIQERSNSKSGD-----FEHFKECIRSTKTYHLFLKHTNTN-----VEYITMAFWAIFSL	354
TaPCS1	LLVLQPNTWLGIKDENVKAEFQSLVSTDNLPDLLKQEIHLHRRQLHYLAGCKGQEAQCQPPSP--	500
ApPCS1	LLALPPQTWSGIKDQALMHMKQLISMASLPTLLQEEVLHRRQLQLLKRCQENKEEDDLAAPAY	485
CePCS	AVAAALLIAWPFKEGYSERSDRIGNLAEKYKNEFSAETMNMSE-----	371
SpPCS	PMIQALPKGVLEEIQSLLEKEVEISEINTQLTALKKQLDLSLTHCCKTDTGCCSSSSCKNT-----	414

Figure 1.1 Alignment of canonical PC synthase amino acid sequences. *A. thaliana* (AtPCS1), *T. aestivum* (TaPCS1), *S. pombe* (SpPCS) and *C. elegans* (CePCS) sequences were aligned using ClustalW2 (www.ebi.ac.uk/clustalw) and edited using Jalview. Conserved catalytic residues and cysteines are boxed. Asterisks denote the catalytic cysteine, histidine and aspartic acid.

Overall, the N-terminal domain shows significant homology to members of the Clan CA cysteine protease family. Particularly, the catalytic cysteine, histidine, and aspartic acid of PC synthases align perfectly with the identical residues in Clan CA cysteine proteases; with the exception that aspartic acid of the former replaces the asparagine of the latter. The three amino acids compose a catalytic triad in which the cysteine is a nucleophilic residue, whose reactivity is dependent upon the donation of its sulfhydryl proton to its neighboring histidine. The catalytic aspartic acid stabilizes the cysteine-histidine pair [4]. The catalytic cysteine has another role in PC synthase catalysis. It is the site of PC synthase acylation by γ -Glu-Cys. The formation of the acyl intermediate is cadmium independent [11].

The C-terminal domains of PC synthases are considered divergent, however, high cysteine content seems to be a commonality. In early studies of PC synthase, it was postulated that the cysteine-rich, C-terminal domain would bind heavy metals or excess essential metals and bring them into contact with the N-terminal end, which was believed to be the catalytic center [17]. However, evidence that the C-terminal domain is not required for enzyme activity *in vivo* or *in vitro* has been well documented [13-15, 18]. Interestingly there is evidence to support that the C-terminal contains a second acylation site (although the exact location of the site is unknown) or is necessary to promote acylation of a second site within the N-terminal. In contrast to the acylation site at the catalytic cysteine, this second site is cadmium dependent [14].

1.3.3 Phytochelatinsynthase activation

There are two models that have been proposed for the activation of PC synthases. In one, the enzyme is activated by metals that directly bind to the enzyme. This theory is supported by the finding that PC synthases contain specific cadmium binding sites [16] and that metal chelators terminate PC synthesis by PC synthase [10,

19]. In the other, the enzyme is activated by S-substituted GSH derivatives. This theory is supported by the fact that under *in vitro* assay conditions almost all of the cadmium in the assay is present as a bis(glutathionato)cadmium ($\text{Cd}\cdot\text{GS}_2$) complex. Additionally, a study of AtPCS1 kinetics revealed that enzyme activity increases with increasing GSH and $\text{Cd}\cdot\text{GS}_2$ concentrations and it has been found that the enzyme can synthesize S-alkylphytochelatins (S-alkyl-PCs) from S-alkylglutathiones (S-alkyl-GSH) in the absence of metal [20]. Both models are discussed in detail in Chapter 3.

1.3.4 Phytochelatin synthase cleavage of GS-conjugates

The main function of PC synthase *in vivo* is to catalyze the synthesis of PCs for either metal homeostasis or detoxification. Notably, PC synthases have also been reported to degrade GSH-S-conjugates (GS-conjugates): an integral part of GS-xenobiotic metabolism [21]. Partially purified PC synthase from *S. cucubalus* and purified AtPCS1 incubated with monobromobimane or monochlorobimane labeled GSH (GS-bimane), resulted in the degradation of the complex to γ -EC-bimane, in a cadmium-dependent reaction [10, 21]. This function was also observed *in vivo* in *A. thaliana* plants grown in media containing monochlorobimane. Wild type *A. thaliana* plants were able to cleave the terminal glycine from the GS-bimane complex, which was synthesized *in vivo* by the enzyme GSH-S-transferase, while mutants lacking PC synthase could not [22].

1.4 OBJECTIVES

In this work the characterization of a PC synthase from the marine diatom *Thalassiosira pseudonana* (TpPCS3) is described. Parallel experiments performed using AtPCS1 are used to compare the requirements for optimal activation of the two

enzymes. A mechanistic study of TpPCS3 and AtPCS1 is also reported. In addition, two PC synthase gene homologues from *T. pseudonana* are cloned and expressed and their subsequent enzymes were assayed in an attempt to determine their functions (see appendix). The results of this work show that all PC synthases are not equal and evidence is provided to suggest that direct metal binding to these enzymes is a requirement for their activation.

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CHAPTER TWO
COMPARISON OF PHYTOCHELATIN SYNTHASES FROM
***THALASSIOSIRA PSEUDONANA* AND *ARABIDOPSIS THALIANA*:**
OPTIMAL CONDITIONS FOR ACTIVATION

2.1 RATIONALE

2.1.1 Introduction

Phytochelatins have been found in a large number of organisms. The discovery of genes encoding for PC synthases has led to the characterization of a few of these enzymes. The kinetics, pH and temperature profiles of PC synthases from a few sources have been individually explored [1-4], but a direct comparison of two different PC synthases using consistent assay conditions has not been published. In this chapter, we compare a PC synthase from a higher plant, *Arabidopsis thaliana* (AtPCS1) to a PC synthase from a marine diatom, *Thalassiosira pseudonana* (TpPCS3). Since its cloning, expression and purification, AtPCS1 has been used as the model PC synthase by several groups interested in elucidating its mechanism [3-10]. Hence, it is currently the most well studied PC synthase. Our interest in PC synthase from *T. pseudonana* stems from studies of the diatom's PC production under laboratory conditions that simulated environmentally relevant metal concentrations. As will be discussed later, this diatom "overproduces" PCs in response to cadmium, when compared with other algae [11].

Because land plants and diatoms differ in both their evolutionary history and current environments, coupled with some unique findings about PC synthesis in *T. pseudonana*, we hypothesized that TpPCS3 and AtPCS1 may have significantly different characteristics.

2.1.2 Evolution of diatoms and higher plants

Diatoms have an evolutionary history that distinctly differs from higher plants [12]. Higher plants, green algae and some red algae arose from an event that was believed to have occurred about 1.5 billion years ago when a eukaryotic heterotroph engulfed a cyanobacterium [13]. Eventually, genes were transferred from the cyanobacterium genome to the nucleus of the host eukaryotic heterotroph, giving rise to the group Plantae [14]. About 500 million years later, a second endosymbiosis occurred, in which a different eukaryotic heterotroph engulfed a photosynthetic red alga. The resulting endosymbiont was eventually transformed into the plastids of Stramenophiles (which include brown macroalgae, plant parasites and diatoms). Genes from the plastids of the Stramenophiles and from the red algae nucleus were transferred to the nucleus of the host, from which the diatoms of today arose. Because of these two separate events, diatoms have many capabilities that differ from higher plants, e.g. the presence of a full urea cycle in diatoms that is absent in higher plants [15, 16].

In addition to their differing evolutionary history, marine diatoms and land plants exist in drastically different environments. While both organisms must acquire trace metals (in particular iron, cobalt, zinc, nickel, and copper), marine phytoplankton have the disadvantage that the availability of metal nutrients in the ocean is much lower than in the soil. Marine diatoms must compete with other phytoplankton for nutrient uptake and cope with the extremely low solubility of metals such as iron at the pH of seawater. Phytoplankton are consumed by zooplankton and are then disposed of in the form of dense zooplankton fecal pellets, resulting in a downward flux of essential nutrients from the ocean surface. Simultaneously, these nutrients are released by heterotrophic bacteria at depth, creating a characteristic profile in which nutrients

are depleted at the surface of the ocean, leaving only picomolar to nanomolar concentrations [17].

To combat these obstacles, it is hypothesized that phytoplankton secrete ligands to more efficiently capture metals. They have also evolved enzymes to help cope with these environmental conditions. A recent example of enzyme adaptation is the discovery of an alternate carbonic anhydrase (CA) in the marine diatom, *Thalassiosira weissflogii*. Unlike most plants and algal CAs that require zinc or cobalt, this particular CA, called CDCA1, can use a cadmium cofactor in its active site to convert bicarbonate (HCO_3^-) to CO_2 by dehydration [18]. Normally, cadmium is deleterious to cells, as it can replace essential metals in some enzymes. But, it was observed that under growth conditions in which zinc concentrations are low, supplemental cadmium could restore *T. weissflogii* growth rates by an increase in CDCA1 activity [18]. Although the major and most catalytically efficient CA in *T. weissflogii* utilizes zinc (or cobalt) in its active site [19], it is believed that the additional activity of CDCA1 could give the organism a competitive edge in ocean survival [20]. CDCA1 genes have been located in the genomes of several marine diatoms including *T. pseudonana*, evidence that these organisms are capable of evolving enzymes to help them adapt to nutrient-limited environments [21, 22].

2.1.3 Phytochelatin synthesis in *Thalassiosira pseudonana* and *Thalassiosira weissflogii*

In laboratory studies of PC synthesis by marine algae, *T. pseudonana* and *T. weissflogii* produced significantly more PCs relative to the proportion of intracellular cadmium than the other algae studied. When grown in media containing the optimal cadmium concentration for PC synthesis, *T. pseudonana* and *T. weissflogii* produced a total γ -Glu-Cys to intracellular cadmium ratio of 80:1 and 100:1, respectively (for PC

chains of length $n = 2, 3$ and 4) [11, 23]. This ratio is 20 to 25 times more than the optimal 4:1 ratio of cysteinyl-sulfur to cadmium determined by EXFAS spectroscopy [24]. About 47% of the γ -Glu-Cys was contributed by PC₂, the peptide of interest in our studies. Thus, the molar ratio of γ -Glu-Cys contributed by PC₂ to intracellular cadmium was 15:1 and 12:1 for *T. weissflogii* and *T. pseudonana*, respectively. Interestingly, a high intracellular PC to cadmium ratio was not found in another member of the *Thalassiosira* genus that was studied, namely *T. oceanica*, suggesting that PC synthesis and possibly PC synthases vary even among closely related species [23].

In this chapter we describe the cloning, expression and purification of AtPCS1 and TpPCS3. Though this work was in part described by Gupton [25], several complications prevented complete characterization of these enzymes. Specifically, difficulty in producing enough TpPCS3 for purification, metal contamination of the purified enzymes, and the dependence of TpPCS3 on a reductant for activity was encountered. In this work, these complications are remedied and TpPCS3 is compared to AtPCS1 in terms of susceptibility to oxidation, optimum pH, optimum temperature and kinetics. Our findings suggest that, although the activation requirements for TpPCS3 and AtPCS1 are the same, the conditions under which each enzyme is optimally activated are different. Our studies also revealed evidence to suggest that along with metal and GSH, the presence of reduced protein thiols are a necessary requirement for PC synthase activity. Additionally, the kinetics of TpPCS3 were consistent with a ternary complex mechanism, which is in contrast to the Ping Pong model that best described the kinetics of AtPCS1 [3].

2.2 METHODS AND MATERIALS

2.2.1 Vector construction and *Escherichia coli* transformation

TpPCS3 and *AtPCS1* were amplified via PCR using vector templates whose construction was described by Gupton [25]. The original source of the genetic material was cDNA generated from reverse transcription polymerase chain reaction performed on *T. pseudonana* and *A. thaliana* lysates as described by Wei [26]. Primers were designed to add the FLAG tag sequence (DYKDDDK) to the 3' end of each gene. In addition, restriction sites NdeI and EcoRI were added to the 5' and 3' ends of *TpPCS3*, respectively. NheI and EcoRI were added to the 5' and 3' ends of *AtPCS1*, respectively. The sequences "ttat" and "aaat" were also added to the respective 5' and 3' ends of each gene as supporting nucleotides for the restriction enzymes to bind to (Table 2.1). *TpPCS3* and *AtPCS1* were cloned into the pET-24a(+) vector (Novagen). The vector was then transformed into the *E. coli* strain BL21(DE3)pLysS (Novagen) according to the manufacturer's instructions. Plasmids from the positive transformants for each gene were sequenced using the vector primer sequences provided by the manufacturer (Bioresources Center, Cornell University). An empty pET-24a(+) vector was transformed into *E. coli* BL21(DE3)pLysS cells to serve as a negative control.

Table 2.1. PCR primers for *TpPCS3* and *AtPCS1* gene amplification. Capital letters denote restriction site sequences.

AtPCS1	
Forward:	5'-ttatGCTAGCatggctatggcgag-3'
Reverse:	5'-ctcgctgctcctgcctatgattataaagatgatgatgataaataataaGAATTCaaat-3'
TpPCS3	
Forward:	5'-ttatCATATGcaaccaccactaac-3'
Reverse:	5'-gatttggatattgattataaagatgatgatgataaataataaGAATTCaaat-3'

2.2.2 Expression of TpPCS3 and AtPCS1

The gene expression protocol was adapted from Romanyuk et al. [8]. Three 33 mL aliquots of Select APS (Alternative Protein Source, Difco) broth were inoculated with 3 mL of overnight culture and grown to an absorbance of 0.6 to 0.8 at OD₆₀₀ at 30°C in a shaking incubator. The cultures were cooled on ice for 5 minutes and then protein expression was induced by the addition of 0.6 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were incubated at 18°C for 9 hours and harvested by 30 minutes of centrifugation at 3000 g and 4°C. To wash the cells, the resulting supernatant was discarded and each pellet was re-suspended in 5 mL of phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.47 mM KH₂PO₄. The re-suspension was distributed evenly into approximately eight to ten 2 mL volume centrifuge tubes and centrifuged at 13,000 rpm and 4°C for 10 minutes. The supernatant was removed and the pellets were placed on ice for lysis preparation.

2.2.3 TpPCS3 and AtPCS1 purification

E. coli cell pellets (in 2 mL volume centrifuge tubes) containing TpPCS3 or AtPCS1 recombinant proteins were re-suspended in 1.5 mL ice cold lysis buffer (Table 2.2). To each re-suspended pellet, 500 µL of 0.1 mm glass beads (Biospec Products) were added. If necessary, additional lysis buffer was added to each pellet re-suspension to minimize air headspace. The tubes were placed on ice and lysed by beadbeating using a Mini-Beadbeater-8 (Biospec Products). Cells were lysed by 5 cycles of beating for 30 seconds (homogenate speed) and then cooling on ice for 2 minutes. The lysed cells were centrifuged at 13,000 rpm for 30 minutes at 4°C. TpPCS3 or AtPCS1 was purified from the resulting crude cell extract supernatant by use of a FLAG M Purification Kit (Sigma Aldrich) according to the manufacturer's

instructions with the exception of the buffer alterations described in Vatamaniuk et al. [10] (Table 2.2). The purification took place in a cold room at 4°C. The purified enzymes were stored at -80°C.

Table 2.2. FLAG M affinity gel purification buffers.

Tris Buffered Saline (TBS)
50 mM tris, 150 mM NaCl, adjusted to pH 7.4 with HCl
Lysis Buffer
50 mM Tris, 150 mM NaCl, 10% glycerol (v/v), 1% Triton (v/v), 2 mM PMSF, adjusted to pH 7.4 with HCL
Elution Buffer
0.1 M glycine, 10% glycerol (v/v), adjusted to pH 3 with HCL
Storage Buffer
50 mM tris, 150 mM NaCl, 50% glycerol, 0.02% sodium azide (w/v), 2 mM PMSF

2.2.4 Protein concentration measurement

Crude cell extract and purified protein concentrations were measured using the Coomassie Plus-The Better Bradford Assay Kit (Pierce) according to the manufacturer's instructions.

2.2.5 Polyacrylamide gel electrophoresis and western blot analysis

For polyacrylamide gel electrophoresis (PAGE) analysis, proteins were separated on 10% denaturing PAGE gels and were stained with Coomassie blue [27]. For western blot analysis, the proteins of unstained gels were transferred onto PVDF membranes as described in Sambrook and Russell [27]. A BioRad MiniProtean Tetra System was used for these procedures. Western blots were probed with a polyclonal anti-FLAG antibody from rabbit (Sigma). The antibody was detected using the WesternBreeze Western Blot and Chromogenic Immunodetection Kit (Invitrogen).

2.2.6 TpPCS3 and AtPCS1 assays

TpPCS3 and AtPCS1 activity was measured in μ moles of PC₂ formed during the reaction per mg of enzyme in the reaction per assay length in minutes. Purified TpPCS3 or AtPCS1 (0.5 or 1 μ g/mL) or crude cell extract containing the enzymes (200 μ g/mL of total protein) was added to substrate buffer containing 200 mM HEPES (pH 8), GSH, and CdCl₂ for three minutes at room temperature unless otherwise specified. After the specified time, a 10 μ L sample of the assay was removed and immediately added to 990 μ L of 10 mM methanesulfonic acid (MSA). The assay samples were stored at -20°C or immediately prepared for HPLC (high performance liquid chromatography) analysis.

2.2.7 TpPCS3 and AtPCS1 anaerobic purification and assays

Anaerobic purification and assays were performed inside a nitrogen/helium filled anaerobic chamber using a palladium catalyst (Coy Laboratories Inc.). Prior to use, buffers were purged with nitrogen gas and equilibrated inside the chamber for at least 24 hours. Assay components such as GSH, dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are susceptible to oxidation and were prepared fresh on a daily basis. These components were reconstituted inside the chamber by the addition of sterile ultrapure water (resistivity 18.2 M Ω ·cm) previously purged with nitrogen gas.

The FLAG M affinity gel was prepared for anaerobic purification according to the manufacturer's instructions with the exception that the column was placed inside the chamber and 20 column volumes of de-oxygenated lysis buffer were used to equilibrate the column prior to the addition of cell lysates.

For TpPCS3 or AtPCS1 purification *E. coli* cell pellets were prepared as described in "TpPCS3 and AtPCS1 purification" inside the anaerobic chamber.

Because the temperature of the chamber could not be cooled to 4°C, 2 mM phenylmethylsulphonyl fluoride, a serine protease inhibitor, was added to the lysis buffer. Parafilm was used to seal the centrifuge tubes, which were then placed on ice and removed from the chamber for the beadbeating procedure and centrifugation, as described in ‘TpPCS3 and AtPCS1 purification’. The cell lysates were returned to the anaerobic chamber and the resulting supernatants were immediately applied to the FLAG M affinity column for the purification procedure.

Purified protein elutant was assayed immediately inside the anaerobic chamber or transferred to a sterile glass vial which could be sealed with an air-tight rubber stopper and crimped metal cap. The purified enzymes were stored at -80°C.

2.2.8 PC₂ quantification

All PC₂ synthesis assay samples were separated by HPLC using a Beckman System Gold 125(S) Solvent Module equipped with an autosampler. Assay samples were derivatized with the fluorescent thiol label monobromobimane (mBBBr, Molecular Probes) and separated on a Supelco-Discovery Reverse Phase Amide-C16 (4.6 x 250) column (Alltech) as described in Wei et al. [28]. Briefly, the PC synthase assay samples were diluted 10 times in 10 mM methanesulfonic acid MSA (making the total assay dilution 1000 fold). The thiols of the sample were reduced by DTT addition. The pH of the sample was raised by the addition of sodium tetraborate and then the mBBBr label was added. The reaction was terminated by the addition of 800 mM MSA. A 100 µL volume of the derivatized sample was injected for HPLC analysis. The HPLC method used to separate the sample components was “Method C” described in Wei et al. [28]. The method was designed to separate low molecular weight thiols including cysteine, γ-Glu-Cys, GSH and PC₂. Fluorescence was detected using a Gilson 121 filter fluorometer (310-410 nM excitation, 475-650 nM emission).

Beckman System Gold 32 Karat software was used to convert the fluorescence signal to a chromatogram and to calculate the peak areas of the separated compounds. A PC₂ standard (Anaspec) was used to create a standard curve.

2.3 RESULTS AND DISCUSSION

2.3.1 Cloning, expression and purification of *TpPCS3* and *AtPCS1*

Early attempts to express *TpPCS3* in *E. coli* for purification purposes resulted in low concentrations of the gene product. *TpPCS3* had been cloned into the vector ptrchis2 (Invitrogen), which had been subsequently transformed into the *E. coli* strain, TOP10F' (Invitrogen). The presence of *TpPCS3* synthesis was only detectable by means of western blot, and one liter of *E. coli* only yielded about 125 µg of enzyme after purification. Attempts to increase protein expression by varying the growth media, temperature, time of induction and concentration of the inducer molecule IPTG used during induction did not significantly increase the amount of *TpPCS3* synthesized. Additionally, the *TpPCS3* transformation reaction yielded no colonies containing the *TpPCS3* gene in the correct orientation, unless the selection plates were supplemented with GSH. This may have been due to the fact that *TpPCS3* used GSH, a peptide required by the cell, as a substrate for PC synthesis. This may have resulted in few colonies containing *TpPCS3* surviving the gene transformation, *TpPCS3* degradation or inclusion body formation of the enzyme during *TpPCS3* expression experiments. It was concluded that the low expression of *TpPCS3* was likely caused by toxicity of the enzyme as well as the intrinsically low expression of the ptrchis2-TOP10F' system [25].

To produce higher concentrations of *TpPCS3*, an expression system that has been engineered for the high expression of potentially toxic proteins was employed. *TpPCS3* and *AtPCS1* were cloned into the Novagen pET-24a(+) vector which was

transformed into *E. coli* strain BL21(DE3)pLysS (Novagen). The BL21 strain is highly compatible with pET vectors and features a chromosomal copy of the gene encoding for T7 RNA polymerase, which, under the control of the LacUV promoter, transcribes the gene of interest. This strain also expresses T7 lysozyme, which suppresses basal expression of T7 RNA polymerase. Suppression of the T7 RNA polymerase, in effect, ultimately suppresses the expression of the gene of interest prior to induction.

Expression of *TpPCS3* and *AtPCS1* using the pET-BL21pLys system proved to be superior to the ptrchis-TOP10F' system. One hundred percent of randomly selected colonies generated from the transformation of *TpPCS3*-pET-24a(+) vectors into BL21pLys cells were positive clones. Over 50 colonies per plate were obtained versus one colony when the ptrchis-TOP10F' system was used. *TpPCS3* and *AtPCS1* synthesis was detectable by PAGE and Coomassie Blue staining. A 100 mL volume BL21 *E. coli* expressing *TpPCS3* resulted in up to 750 µg of enzyme after purification. The purification of both enzymes was confirmed by PAGE and western blot (Figure 2.1). Both electrophoresis and blotting procedures yielded a single band for *TpPCS3* (54 kDa) and a single band for *AtPCS1* (58 kDa) that corresponded to the molecular weights predicted for each enzyme plus the size of the FLAG tag.

An alignment of the *TpPCS3* amino acid sequence with other canonical PC synthase protein sequences showed that *TpPCS3* contains the features unique to all PC synthases: a highly conserved N-terminal domain, a divergent cysteine rich C-terminal domain, and the conserved catalytic triad amino acids (cysteine, histidine and aspartic acid) as shown in a alignment of *TpPCS3* with canonical PC synthases (Figure 2.2) [29].

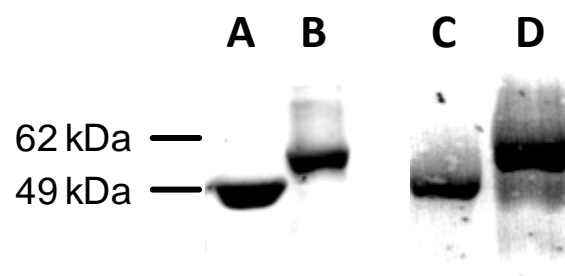


Figure 2.1. Purification and immunoaffinity detection of TpPCS3 and AtPCS1. For PAGE analysis, purified enzymes (1 μ g) were stained with Coomassie Blue (A) TpPCS3 and (B) AtPCS1. For western blot analysis, purified enzymes (2.5 μ g) were probed with an anti-FLAG antibody. (C) TpPCS3 and (D) AtPCS1. Sizes of a molecular weight standard run in parallel are indicated.

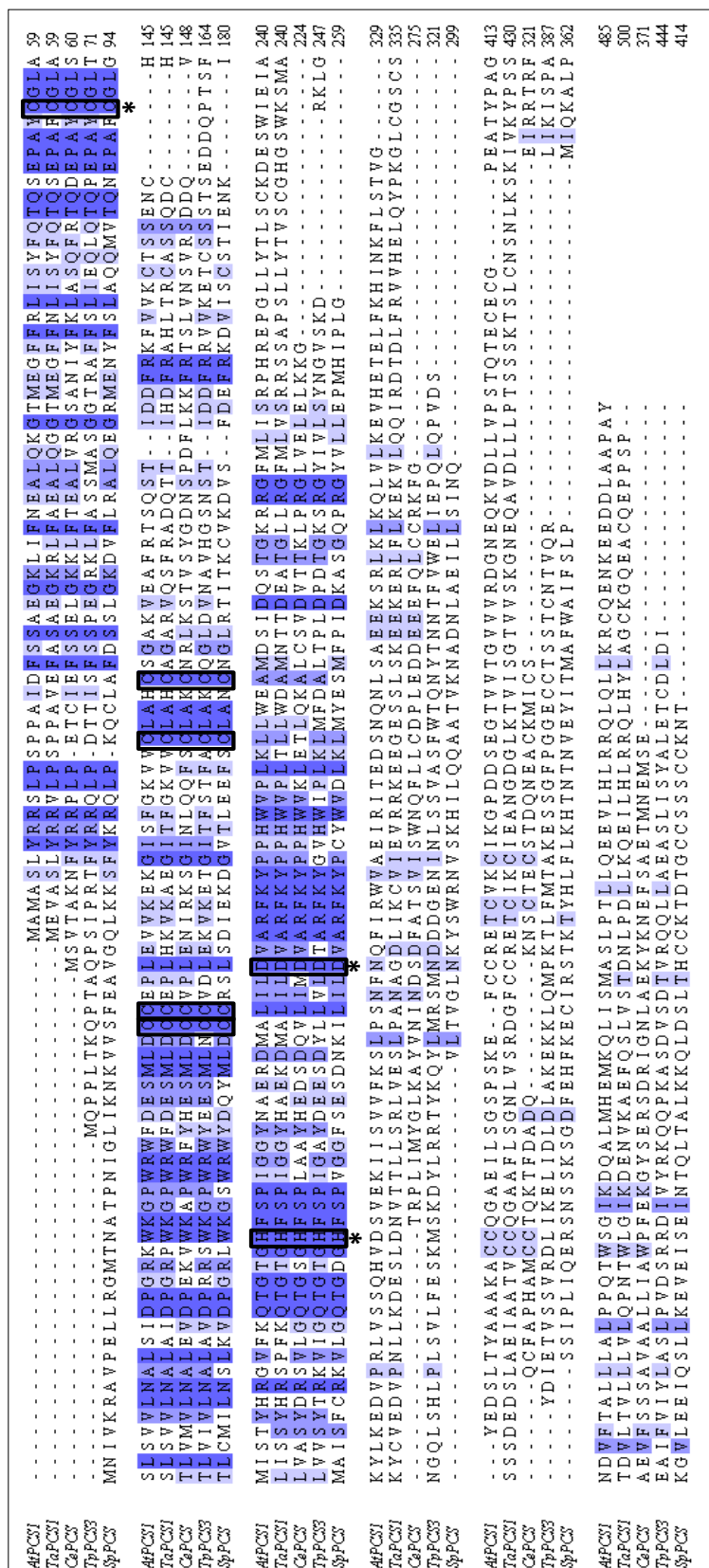


Figure 2.2. Amino acid sequences of canonical PC synthases aligned with TpPCS3. TpPCS3, AtPCS1, TaPCS1, CePCS and SpPCS sequences were aligned using ClustalW2 (www.ebi.ac.uk/clustalw) and edited using Jalview. Conserved cysteines and catalytic residues are boxed. Asterisks denote the catalytic cysteine, histidine and aspartic acid.

2.3.2 The effects of reductants on aerobically purified TpPCS3 and AtPCS1 activity

As reported in Gupton [25] and in agreement with Vatamaniuk et al. [10], purified AtPCS1 was active in substrate buffer containing GSH and cadmium. Conversely, as also discussed in Gupton [25], purified TpPCS3 did not display PC₂ synthesis activity following purification unless 5 mM DTT, a reductant was added to the substrate buffer. DTT contains two thiol groups in close proximity to each other. Upon the donation of their protons, the sulfurs of these thiols form a stable intermolecular disulfide bond which results in a stable ring formation, making DTT a strong reducing agent [30]. Along with their reducing capability, thiol groups are also strong chelators of soft metals such as cadmium and copper, therefore, DTT acts as both a reductant and a metal chelator.

Because of the dual action of DTT, the activation of TpPCS3 by this reductant in assays could be explained by two potential scenarios. (i.) The formation of disulfide bonds renders TpPCS3 inactive (or less active) by altering its secondary or tertiary structure. Alternatively, the active site cysteine of TpPCS3 has become oxidized and no longer contains a proton to donate to the histidine of the catalytic triad. As mentioned, this is a necessary condition for PC synthase activation. In this case, DTT reduces the disulfide bonds or protonates the active site cysteine, allowing activation of the enzyme. (ii.) At the cadmium concentrations used in the assay, TpPCS3 is inactivated by “metal poisoning” which may occur if metal binds to the sulfur of the active site cysteine. The supplemental DTT remedies this problem by chelating the excess cadmium, resulting in enzyme activation.

In an attempt to determine the method of PC synthase activation by DTT, we varied the concentration of the reductant in the TpPCS3 and AtPCS1 assays. We also compared the effects of adding two other reductants: tris (2-carboxyethyl) phosphine hydrochloride (TCEP), which is also a strong reductant that does not contain thiol

groups, and 2-mercaptoethanol (2-ME) which contains one thiol and is commonly used in biochemical assays (Figure 2.3).

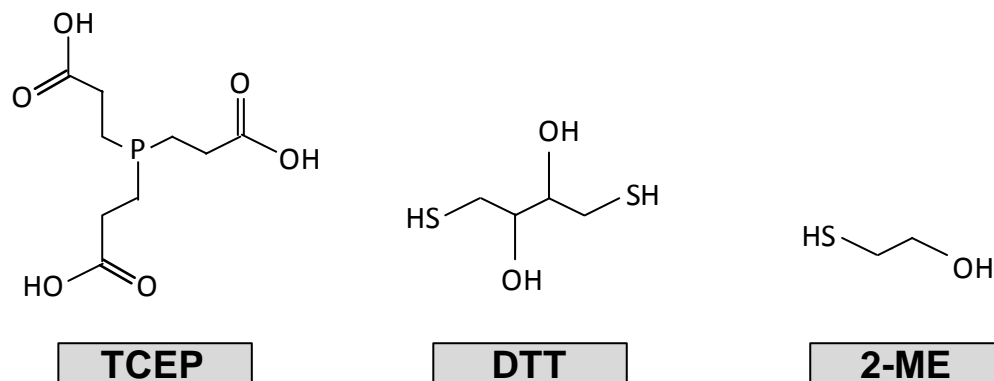


Figure 2.3. Molecular structures of reductants. A) dithiothreitol (DTT) B) tris (2-carboxyethyl) phosphine hydrochloride (TCEP) C) 2-mercaptoethanol (2-ME).

In agreement with Gupton [25], TpPCS3 activity increased with increasing concentrations of DTT and TCEP. At the highest concentration tested (15 mM), DTT and TCEP were 7 times more effective than 2-ME at TpPCS3 activation. Activation of TpPCS3 by 2-ME was minimal, although detectable (Figure 2.4 A). In the case of AtPCS1, the addition of 5 mM TCEP to the assay more than doubled its activity, while increasing DTT concentrations decreased enzyme activity at concentrations higher than 5 mM. The addition 5 mM and 10 mM 2-ME to AtPCS1 assays had no effect on activity, however, the addition of 15 mM 2-ME nearly doubled the activity of the enzyme (Figure 2.4 B).

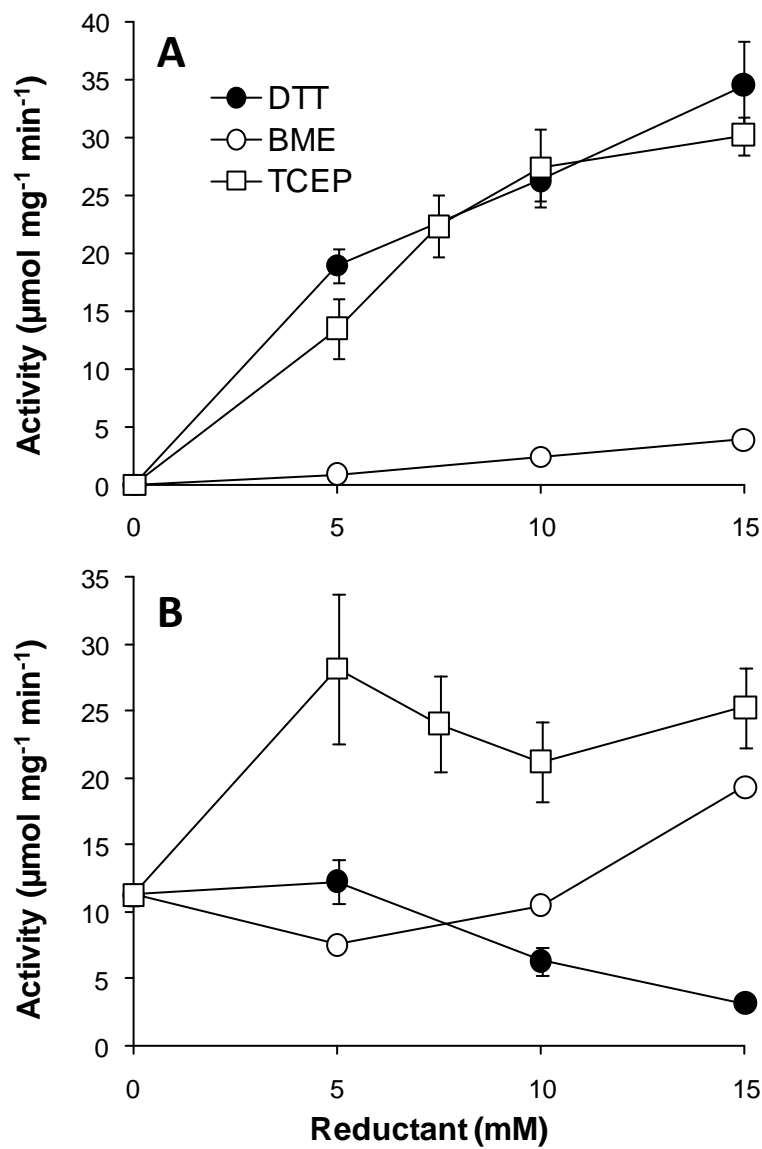


Figure 2.4. Effect of reductants on purified TpPCS3 and AtPCS1 activities. TpPCS3 (A) and AtPCS1 (B) (1 $\mu\text{g/mL}$) were assayed in substrate buffer containing 7 mM GSH, 50 μM CdCl_2 , and reductant as indicated. Assay length was 3 minutes. Error bars represent means \pm S.E. ($n = 3-4$). Where not shown, error bars are within symbol.

Explanation (ii), that DTT protects TpPCS3 from metal poisoning, can be ruled out since TCEP was able to activate TpPCS3 in a similar manner as DTT although the stability constant ($\log \beta$ (ML)) for TCEP to cadmium (5.00) [31] is almost 3 times lower than that of DTT to cadmium (14.64) [32]. Since the activity of AtPCS1 was increased by TCEP addition, it can be assumed that this enzyme was also partially oxidized. This suggests that to some degree, TpPCS3 and AtPCS1 have both been inactivated by reversible oxidation during purification.

With metal toxicity eliminated as a possible explanation for TpPCS3 inactivation, the differential response of TpPCS3 and AtPCS1 to DTT addition can be explained in terms of protein-thiol oxidation of the enzymes and substrate availability as it relates to the concentration of Cd·GS₂. To address substrate availability, MINEQL+, a chemical equilibrium modeling program, was used to predict the actual concentrations of Cd·GS₂ for the assay conditions of 200 mM HEPES-NaOH (pH 8), 50 μ M CdCl₂, 7 mM GSH and the DTT concentrations used in the assays. The presence of DTT in the assays drastically alters the Cd·GS₂ concentration. As predicted by MINEQL+ the concentrations were 0.084 μ M, 0.073 μ M and 0.027 μ M Cd·GS₂ for substrate buffers containing 5 mM, 10 mM, and 15 mM DTT, respectively. These concentrations are extremely low when compared to 50 μ M Cd·GS₂ predicted for assays in which no DTT is added.

We hypothesized that AtPCS1 requires more Cd·GS₂ for maximum activity than is present in the DTT supplemented assays. Essentially, DTT binds the cadmium, preventing the formation of Cd·GS₂ that is necessary as a substrate for AtPCS1. At the same time, DTT is reducing the partially oxidized enzyme, resulting in an increase in its activity. The result of these two opposing actions is no net change in AtPCS1 activity at the 5 mM DTT concentration. At greater concentrations, DTT binds even more cadmium, which lowers the Cd·GS₂ concentration so much that there is

insufficient substrate for enzyme activity. Therefore, although DTT is an effective reductant, enzyme activity decreases with increasing DTT concentrations. The addition of more cadmium to AtPCS1 assayed in substrate buffer containing DTT, did not allow the enzyme to overcome the effects of DTT as a chelator. In fact, when AtPCS1 was assayed in substrate buffer containing 5 mM DTT, and 6 mM GSH, and varying CdCl₂ concentrations, the enzyme activity increased with increasing cadmium up to 50 µM. At 100 µM CdCl₂ the enzyme activity was inhibited (Data not shown).

We hypothesized that TpPCS3 requires less Cd·GS₂ than AtPCS1 for maximum activity. Since all of the enzyme's activity is lost during the purification step, any recovery of enzyme activity will have a quantitatively dramatic effect as long as the substrate concentration is sufficient for activation. Although the addition of DTT decreases the amount the Cd·GS₂ substrate in the assay, the reductant is, in effect, binding excess cadmium that is not required for maximum enzyme activation. Therefore, TpPCS3 activation by DTT is strictly based upon the reducing power of the reductant, i.e. enzyme activity increases with increasing DTT.

The relative ineffectiveness of 2-ME to increase the activity of TpPCS3 is not clear, especially since it seems to increase the activity of AtPCS1. It is possible that 2-ME is a weak reductant when compared to DTT and TCEP, and it is unable to rescue the completely oxidized TpPCS3.

2.3.3 Oxidation sensitivity of TpPCS3 crude cell extract

A complete protein purification consisting of column loading, column washing and elution usually takes about 12 hours to complete since cell extracts and buffers pass through the FLAG affinity column via gravity. The proteins of interest are exposed to air for this period of time, making these conditions conducive to protein

oxidation. In addition, reductants are not compatible with the FLAG tag column since they can reduce the disulfide bridges of the FLAG antibody.

To evaluate the sensitivity of TpPCS3 to oxidation, *E. coli* cell pellets containing TpPCS3 were prepared for lysis inside an anaerobic chamber. The tubes containing the pellet re-suspensions were sealed as tightly as possible with Parafilm.

Lysis of the pellets by beadbeating and centrifugation of the resulting lysate was performed outside of the chamber. The cell-free lysate was returned to the chamber and immediately assayed. Then, the cell extract was removed from the anaerobic chamber and assayed at two time points: 5 minutes and 1 hour. TpPCS3 lost 50% of its activity in the first 5 minutes of exposure to ambient air. The activity of the enzyme remained constant after 1 hour. These results suggested that TpPCS3 is highly susceptible to oxidation and led to the development of a method to purify the enzyme under anaerobic conditions.

2.3.4 The effects of reductants on anaerobically purified TpPCS3 and AtPCS1 activity

TpPCS3 and AtPCS1 were purified inside an anaerobic chamber and their activities were immediately assayed inside the chamber. TpPCS3 activity was nearly four times higher than the highest activity that TCEP or DTT could elicit from the oxidized form of the enzyme. The addition of 5 mM DTT, 2-ME or TCEP to anaerobically purified TpPCS3 assays increased enzyme activity by 34%, 4.4% and 17.3%, respectively. The addition of 5 mM DTT to anaerobically purified AtPCS1 assays had virtually no effect on its activity, while the addition of 5mM 2-ME or 5 mM TCEP increased the enzyme's activity by 40% (Figure 2.5).

Although measures were taken to reduce the amount of oxygen that the enzymes were exposed to during the purification process, the addition of reductants to

both enzymes still increased their activities. This is likely because the intracellular environment of *E. coli* is not completely reducing, and because the centrifuge tubes containing the cell pellets were unable to be completely sealed during the lysis procedure. Nevertheless, the anaerobic purification process described in this work proved to be effective enough to preserve the activity of TpPCS3 and eliminated the need to use reductants in enzyme assays.

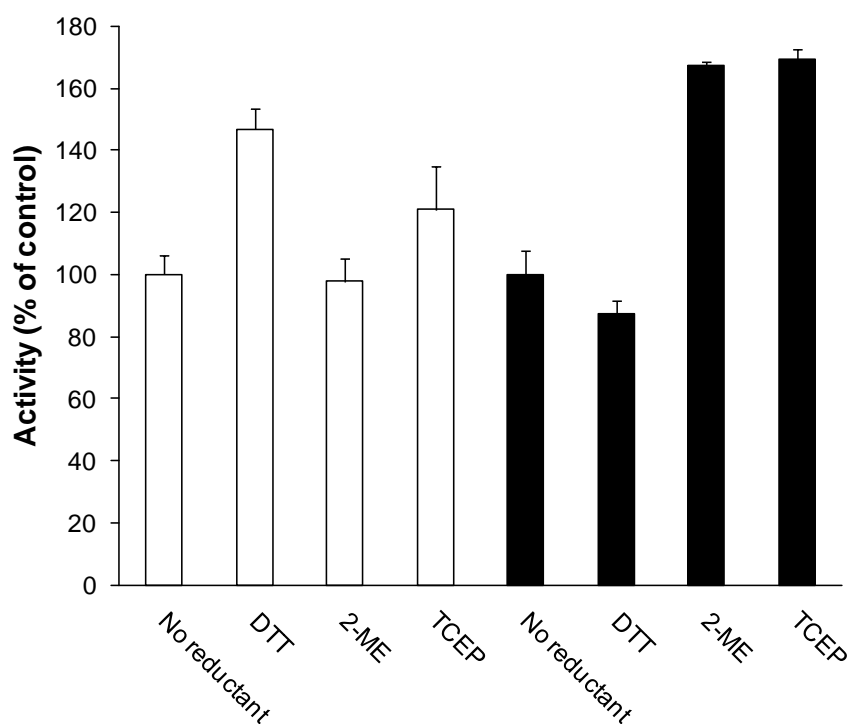


Figure 2.5. Effect of reductants on anaerobically purified TpPCS3 and AtPCS1 activities. Following anaerobic purification TpPCS3 (white bars) and AtPCS1 (black bars) (1 $\mu\text{g/mL}$) were assayed inside an anaerobic chamber in substrate buffer containing 7 mM GSH, 50 μM CdCl_2 and 5 mM reductant where indicated. Activities are presented as percentages of activities measured for each respective enzyme in the absence of a reductant which were 122.4 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ and 38.9 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ for TpPCS3 and AtPCS1, respectively. Error bars represent means \pm S.E. ($n = 3$).

To compare the oxidation rates of purified AtPCS1 and TpPCS3, the enzymes (18 µg/mL) and substrate buffers were removed from the chamber and assays were performed with 1 (µg/mL) at several times over a period of 9 hours at room temperature. TpPCS3 and AtPCS1 maintained their initial activity levels for the first 30 minutes and 4 hours of ambient air exposure, respectively. After these time points, the activity of both enzymes decreased with increasing time. TpPCS3 oxidized more quickly than AtPCS1, losing about 44% of its activity within the first 4 hours. After 9 hours, TpPCS3 had lost 86% of its original activity. AtPCS1 lost 54% of its original activity after 9 hours (Figure 2.6). Since the purification process takes about 12 hours, it is likely that TpPCS3 is completely oxidized and AtPCS1 is partially oxidized during that time. The addition of 5 mM TCEP to TpPCS3 assays after 9 hours of air exposure resulted in the recovery of enzyme activity to 90.36% of its original activity, which supports the conclusion of oxidation over protein degradation.

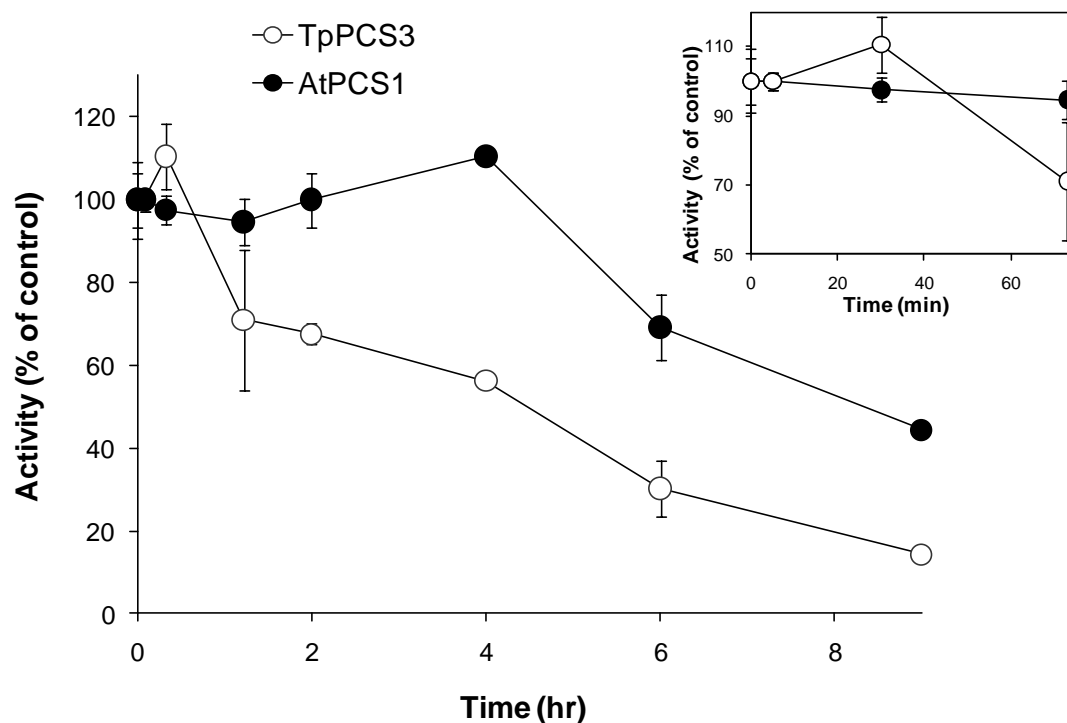


Figure 2.6. TpPCS3 and AtPCS1 time dependent oxidation. AtPCS1 and TpPCS3 (1 $\mu\text{g/mL}$) were assayed inside an anaerobic chamber in substrate buffer containing 7 mM GSH and 50 μM CdCl_2 ($t = 0$). The enzymes were removed from the chamber and assayed after the indicated times had passed. Activities are presented as the percentage of activities measured for each respective enzyme assayed inside the chamber which were $122.4 \pm 8.2 \mu\text{mol mg}^{-1} \text{min}^{-1}$ and $38.9 \pm 2.8 \mu\text{mol mg}^{-1} \text{min}^{-1}$ for TpPCS3 and AtPCS1, respectively. Inset: Expanded view of the first 4 time points. Error bars represent means \pm S.E. ($n = 3$).

2.3.5 Non-reducing PAGE analysis of aerobically purified TpPCS3 and AtPCS1

We hypothesized that the presence of cysteines in TpPCS3 may provide an explanation for its oxidation sensitivity. The thiol groups of cysteines are very redox active and can be modified by covalent reversible reactions. Thiol groups can be oxidized to different oxidation states by various types of oxidizers. However, in this case it was hypothesized that the oxidation of the thiol groups of TpPCS3 resulted in the formation of disulfide bonds which, subsequently, terminates enzyme function. To test whether or not disulfide bond formation affects TpPCS3 and AtPCS1 activity, the aerobically purified enzymes were prepared for PAGE without the addition of 100 mM DTT to the sample buffer. The enzymes were run on a 10% SDS denaturing gel. The electrophoretic mobility of each enzyme was altered when DTT was omitted from the sample buffer. Aerobically purified TpPCS3 ran as a protein with a molecular weight of 98 kDa, which is almost double the molecular weight of reduced TpPCS3 (54 kDa) (Figure 2.1). Two bands were visualized for aerobically purified AtPCS1. One band ran as a 58 kDa protein, the same size as purified AtPCS1 that had been reduced prior to gel loading. The second band ran as a protein slightly larger than 98 kDa, almost twice the molecular weight of reduced AtPCS1 (Figure 2.7). The results of the gel suggest that oxidation of both AtPCS1 (partially) and TpPCS3 (completely) results in the formation of disulfide bonds within the enzymes that alter either the secondary structure (through intermolecular disulfide bonding) or quaternary structure (through intramolecular disulfide bonding). This ultimately diminishes enzyme activity (in the case of AtPCS1) or terminates enzyme activity (in the case of TpPCS3).

Interestingly, a phenomenon similar to the AtPCS1 results presented in this section was reported by Grill et al. [1] as a result of efforts to purify a PC synthase from *Silene cucubalus* cell extracts. In early purification steps an active enzyme

having a 95 kDa molecular weight was detected. In a later purification step active enzymes of size 95 kDa and 50 kDa were separated. Finally, denaturing PAGE analysis yielded a protein of 25 kDa molecular weight. The authors hypothesized that this PC synthase existed as a 95 kDa tetramer consisting of four 25 kDa subunits. Furthermore, they believed that this tetramer dissociated into two active dimers having 50 kDa molecular weights.

The data reported here does not fit this theory exactly since our results suggest that the oxidized form of AtPCS1 is not active, however, both studies seem to support the idea that PC synthases may exist as complex enzymes containing subunits that may somehow affect enzyme activity.

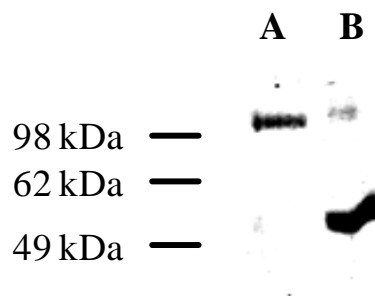


Figure 2.7. Non-reducing PAGE of aerobically purified TpPCS3 and AtPCS1. (A) TpPCS3 and (B) AtPCS1.

2.3.6 Optimal pH, temperature and metal activation requirements for TpPCS3 and AtPCS1

The pH and temperature profiles for PC synthase from *S. cucubalus* [1] and *Lycopersicon esculentum* [2] have been published. In addition, the optimal pH and temperature for AtPCS1 and for a homo-PC synthase from *Glycine max* (GmhPCS1) have been reported [4]. In each case the optimal temperature reported was 35°C and the optimal pH was 8. We compared the pH and temperature profiles for TpPCS3 and

AtPCS1. TpPCS3 and AtPCS1 were both found to have an optimal pH of 8 (Figure 2.8 A). The pH profile for AtPCS1 was similar to the published profiles for PC synthases from *S. cucubalus* and *L. esculentum*. Like these enzymes, AtPCS1 displayed virtually no activity below pH 5 or above pH 10. The pH profile for TpPCS3 only differed from the AtPCS1 profile in that at pH 10 the enzyme maintained over half of its maximum activity.

Different optimal temperatures were determined for AtPCS1 and TpPCS3. AtPCS1 had an optimum temperature range of 25°C -40°C, whereas TpPCS3 activity peaked at 45°C (Figure 2.8 B). The shape of the temperature profile for AtPCS1 was non-traditional. The activity of AtPCS1 increased sharply between 15°C and 25°C. Activity remained constant from 25°C to 40°C and then began to decrease at temperatures higher than 40°C. The reason for the plateau between 25°C to 40°C is unknown. The shape of the TpPCS3 temperature profile was more traditional. The activity increased linearly from 15°C to 45°C then substantially decreased at 50°C.

An optimum pH of 8 for both enzymes was expected since it is likely that the pH of the cytosol in both *A. thaliana* and *T. pseudonana* is similar. However, the optimum temperature for TpPCS3 cannot be explained. For reasons unknown, the structure of the TpPCS3 enzyme appears to be slightly more stable at higher temperatures than AtPCS1.

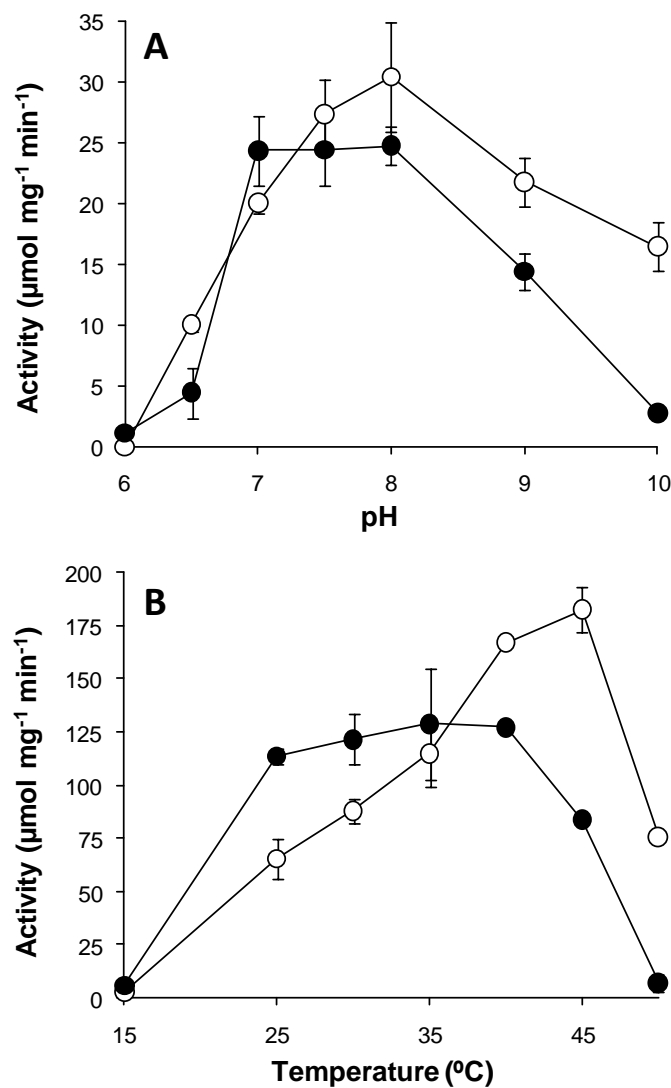


Figure 2.8. TpPCS3 and AtPCS1 pH and temperature profiles. Enzymes (0.5 $\mu\text{g/mL}$) were assayed in substrate buffer containing 6 mM GSH, 50 μM CdCl_2 (AtPCS1) or 2 μM CdCl_2 (TpPCS3). Assay time length was 3 minutes. Error bars represent means \pm S.E. ($n = 3$). (A) pH profile: 200 mM HEPES was adjusted to indicated pH with potassium hydroxide. (B) Temperature profile: substrate buffer was pre-warmed to indicated temperature prior to enzyme addition.

To determine if TpPCS3 and AtPCS1 differed in their activation by various metals, both enzymes (0.5 µg/mL) were assayed in substrate buffer containing 3.3 mM GSH and 50 or 2 µM metal salt (CdCl₂, CuSO₄, ZnSO₄ or CoCl₂) for AtPCS1 and TpPCS3, respectively. The enzymes were assayed at these specific metal concentrations because these concentrations were found to be saturating in our kinetic study (which will be discussed later). The order of metal stimulation for both enzymes from greatest to lowest was CdCl₂, CuSO₄, ZnSO₄ or CoCl₂, consistent with what was reported by Vatamaniuk et al. [3] (data not shown).

2.3.7 TpPCS3 and AtPCS1 kinetics

Kinetic constants were calculated for TpPCS3 by Gupton [25]. However, because TpPCS3 required a reductant for activation, 5 mM DTT was added to the assays. This altered the Cd·GS₂ concentration in the assays since DTT also acted as a chelator. MINEQL+ was used to calculate the actual concentrations of Cd·GS₂ in each assay. The result was that the Cd·GS₂ concentrations adjusted for DTT addition fell into the 0 to 2 µM range. The plot of TpPCS3 activity versus the calculated Cd·GS₂ concentrations at various GSH concentrations yielded a single curve. We assumed that this single curve represented the initial velocity of the enzyme, which appeared to be solely dependent on Cd·GS₂ concentration since that substrate became the limiting factor. Thus, the amount of free GSH in assays did not appear to affect enzyme activity at low Cd·GS₂ concentration. A Lineweaver-Burk plot generated from this curve was used to calculate the K_m of the Cd·GS₂ substrate. The K_m calculated for Cd·GS₂ for TpPCS3 was 0.03 µM, 2 orders of magnitude lower than the K_m published for AtPCS1 (9.1 ± 2.3 µM) [3].

The previous results encouraged us to repeat the kinetic experiments without the use of DTT. At the same time, background metal contamination was removed from

the enzymes as well. Briefly, this was done by first incubating the enzymes with bathocuproine, which is a specific copper(I) chelator, and TCEP. The bathocuproine and TCEP were removed via dialysis against trace metal cleaned HEPES buffer. This process will be discussed in detail in Chapter 3. To create kinetic curves for TpPCS3, the enzyme was assayed in substrate buffer containing varying concentrations of GSH and CdCl₂. Originally, we attempted to determine the kinetic variables by using the Ping Pong Bi Bi System model [33], the model which describes the kinetics of AtPCS1 [3]. This model describes an enzyme mechanism in which one substrate interacts with the enzyme resulting in the release of a product and the formation of an enzyme intermediate. A second substrate then binds to the enzyme resulting in the formation and release of a second product and the return of the enzyme to its original state. The primary and secondary plots of the kinetic data for TpPCS3 revealed that the model that best describes the enzyme is actually the ternary complex model. In this model, two substrates must bind to the enzyme at the same time before the product is synthesized and released. No intermediate is formed. This implies that the active site of TpPCS3 must be able to accommodate both the GSH and Cd·GS₂ substrates at the same time, or that the enzyme contains a second binding site specific for one of the two substrates that must be occupied in order for catalysis to occur. This is in contrast to the proposed activation model for AtPCS1, where the sequential steps of the reaction all take place at one active site. For TpPCS3, the K_M values calculated for Cd·GS₂ and GSH were $0.12 \pm 0.02 \mu\text{M}$ and 37.9 mM , respectively. The v_{max} was $416.7 \mu\text{mol mg}^{-1}\text{min}^{-1}$ (Figure 2.9).

To ensure that our methods were comparable to those used to calculate the K_M for Cd·GS₂ for AtPCS1 in Vatamaniuk et al. [3], one kinetic curve for AtPCS1 was generated at 6 mM GSH. The Cd·GS₂ K_M for AtPCS1 was determined to be 2.4 μ M (Figure 2.10).

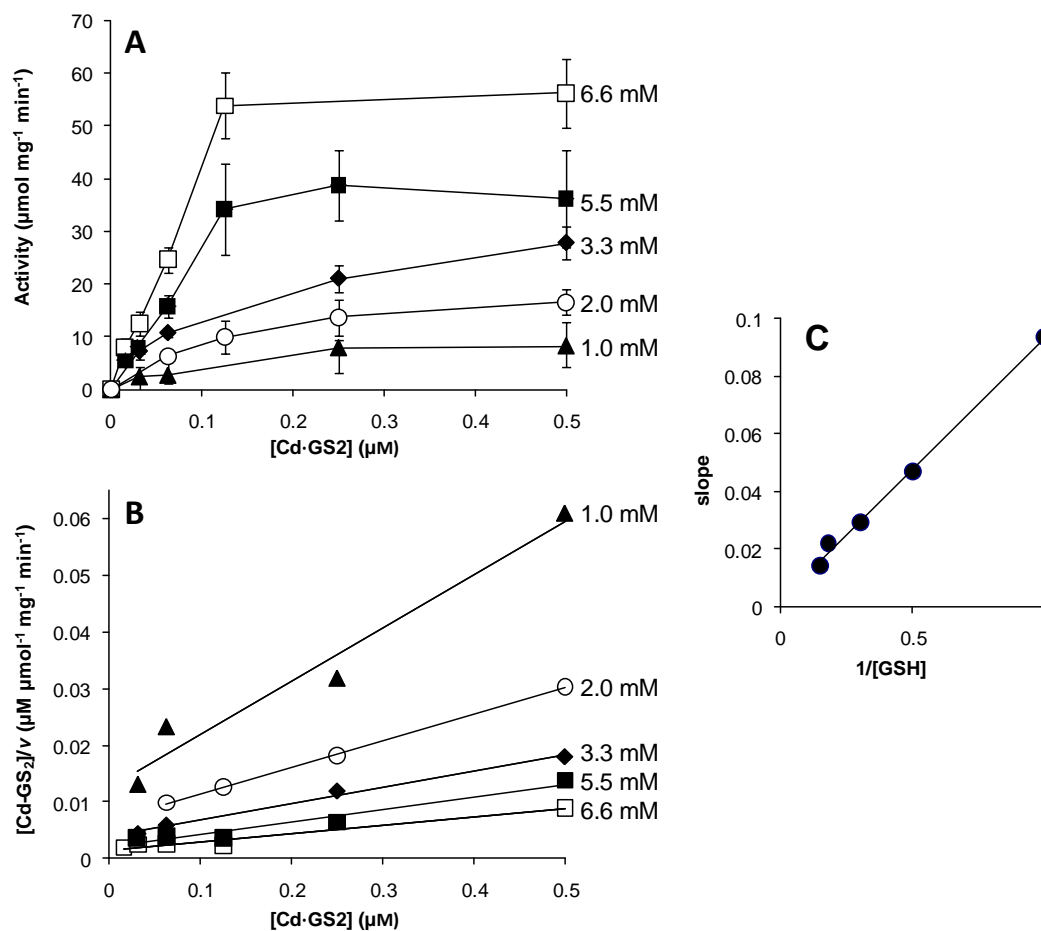


Figure 2.9. Determination of TpPCS3 kinetic variables. (A) TpPCS3 (0.5 μ g/mL) was assayed for 3 minutes in substrate buffer containing varying concentrations of CdCl₂ and GSH. Error bars represent means \pm S.E. ($n = 3-4$). (B) Hanes-Woolf plot for determining the K_m for the Cd·GS₂ substrate. The average of the x-intercepts equals $-K_M$. The R^2 value for each regression line was greater than 0.96. (C) The slopes of the regression lines in (B) were plotted vs. $1/[\text{GSH}]$ to determine the K_M for GSH and v_{max} . The x-intercept equals $-(1/K_M)$ and the y-intercept equals $1/v_{\text{max}}$.

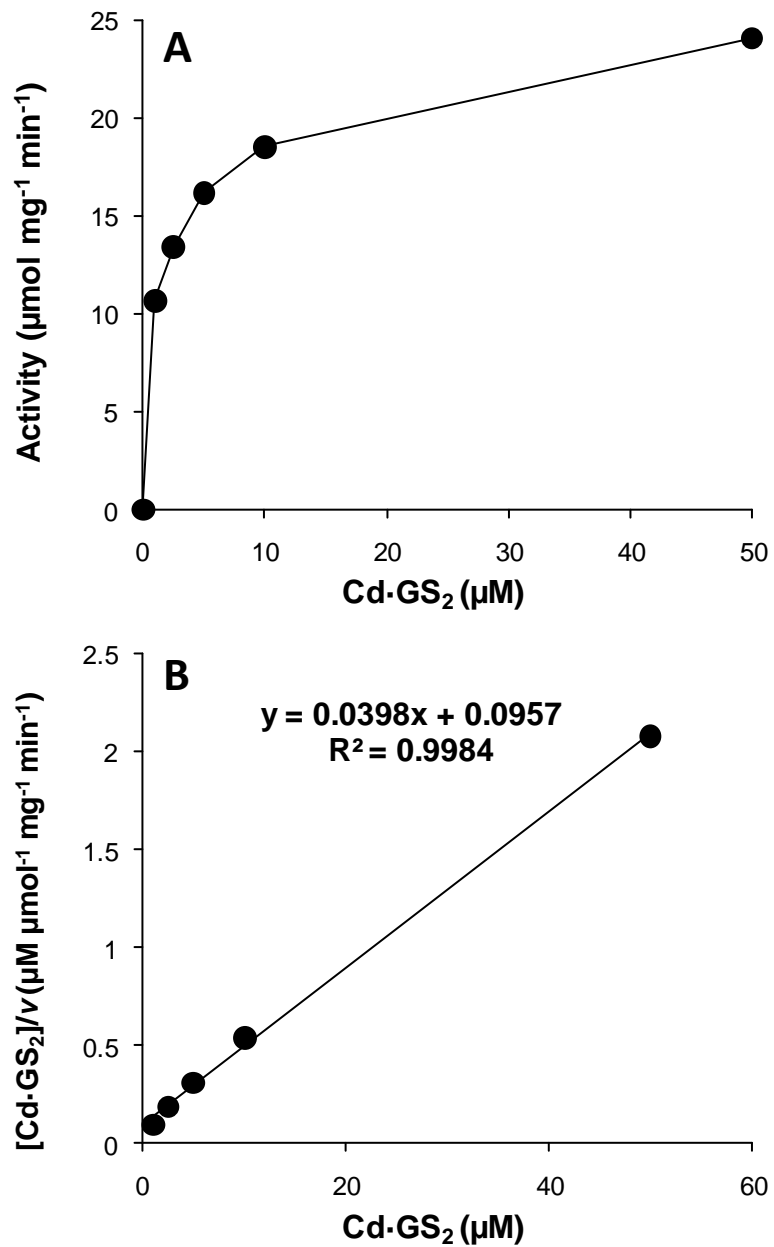


Figure 2.10. Determination of AtPCS1 K_M for Cd·GS₂. (A) AtPCS1 was assayed in substrate buffer containing 6 mM GSH and CdCl₂. (B) The K_M for the Cd·GS₂ substrate was determined by creating a Hanes-Woolf plot. The x-intercepts equals $-K_M$.

2.4 CONCLUSION

Our comparison of PC synthases from a plant and an algal source provided us with two conclusions regarding these enzymes. First, the finding that both TpPCS3 and AtPCS1 are susceptible to oxidation that results in a change in enzyme structure and a decrease in activity suggests that these enzymes require reduced thiols for function. The fact that a common characteristic of PC synthases is the presence of conserved cysteines in the N-terminal domain, coupled with a cysteine rich C-terminal domain, directed our attention toward the possibility that oxidation of these amino acids are responsible for this phenomenon. In AtPCS1, the loss of the cysteine rich C-terminal domain has been shown to decrease activity of the enzyme, but not abolish it entirely [7]. In addition, amino acid substitutions of the N-terminal domain conserved cysteines (with the exception of the catalytic cysteine) did not affect the activity of AtPCS1 [6]. Conversely, in SpPCS, mutation of one of the N-terminal conserved cysteines, has been shown to terminate enzyme activity [5]. It would seem that PC synthases vary in their dependence on the conserved cysteines in the N-terminal domain and therefore would exhibit differential sensitivity to oxidation as was observed in this study. Although this study does not prove that conserved cysteines contribute to PC synthase activation, our data suggests that reduced thiols are a requirement for TpPCS3 activity. Whether or not the deactivation of TpPCS3 by thiol oxidation is a result of a change in structure by inter- or intramolecular disulfide bond formation will require further investigation.

Second our comparison of TpPCS3 and AtPCS1 pH profiles, temperature profiles, metal activation and kinetics showed that activation of PC synthases are likely to be similar in terms of their optimum pH and temperature since these values are similar for all the PC synthases studied from different organisms. However, it should be taken into account that few PC synthases overall have been characterized,

and there is a possibility that more dramatic variations in the optimal activation environments exists for some of the enzymes. Perhaps the most interesting finding in this study was the very different K_M values for Cd·GS₂ calculated for TpPCS3 and AtPCS1. It seems that these enzymes differ the most in their adaptation to intracellular concentrations of essential and toxic heavy metals which are dictated by the natural external environment of the organism. The fact that the K_M value for Cd·GS₂ for TpPCS3 is an order of magnitude lower than that of AtPCS1 may very well be attributed to adaptation of each enzyme to its current environment.

Third, we found that the kinetics of TpPCS3 are best described by the ternary complex model, suggesting that the mechanism of this enzyme may be different from that of AtPCS1. Since amino acid sequence alignments indicate that the active sites of AtPCS1 and TpPCS3 are the same, it is likely that TpPCS3 contains this active site and a second binding site for cadmium or Cd·GS₂. Interestingly, in a study of AtPCS1 acylation, it was shown that this enzyme contains a second cadmium-dependent acylation site that enhances enzyme activity, but is not crucial for catalysis [6]. It is believed that this second site along with a specific cadmium binding site exists in the C-terminal domain. In future studies of TpPCS3, it would be useful to determine if the enzyme forms a cadmium independent acyl intermediate and if it requires its C-terminal domain for activation. The lack of a cadmium-independent acyl intermediate formation and the requirement of a C-terminal domain would not only support our ternary complex data, but it would also indicate that the substrate requirements for TpPCS3 are different than those of AtPCS1.

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CHAPTER THREE
DETERMINATION OF THE ROLE OF DIRECT METAL BINDING TO PC
SYNTASES FROM *THALASSIOSIRA PSEUDONANA* AND *ARABIDOPSIS*
THALIANA

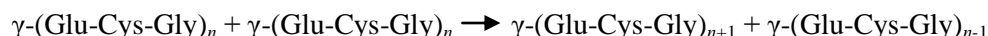
3.1 RATIONALE

3.1.1 Introduction

The elucidation of the mechanism of PC synthase has been an area of active research for more than a decade. It is generally accepted that PC synthases are dipeptidyl transpeptidases that perform two reactions: the first being the degradation of GSH to γ -Glu-Cys and glycine, and the second being the transfer of γ -Glu-Cys to a GSH or PC_n peptide. It is also accepted that the addition of certain metals (e.g. cadmium, copper, mercury, lead and zinc) can induce PC synthesis *in vivo* as well as in *in vitro* assays that mimic physiological conditions [1-3]. However, the specific role that metals play in PC synthase activation is still a subject of debate. This is because metals bind to both the thiol group of the GSH substrate and the thiol groups of cysteines or other metal binding residues such as histidines in the enzyme. Attempts to elucidate the mechanism of PC synthase have been published by a few groups [2, 4-11]; however, the results have often been conflicting. Based upon a review of these studies, we designed experiments in an attempt to increase the understanding of the mechanism, which will be described in the Results section. This introduction provides a brief summary of the experimental results previously published by groups studying the mechanism of PC synthase.

3.1.2 Pioneering studies of phytochelatin synthase

The initial mechanism studies of PC synthase were reported by Grill et al. [2]. Using a PC synthase partially purified from *Silene cucubalus* cell extracts, these investigators reported that PC synthase required two substrates for PC₂ synthesis: GSH and metal. They also reported that longer PC chains could be formed from other PC_n peptides, as is described in the proposed reaction:



In 1999, a PC synthase (i.e. AtPCS1) was first cloned, expressed and purified from *Arabidopsis thaliana*. In this study, the ability of the enzyme to bind cadmium was documented. The binding capacity and ligand binding constant (K_L) for cadmium binding to AtPCS1 was determined to be 7.09 ± 0.94 moles of cadmium per mole of enzyme and 0.54 ± 0.21 μM , respectively [5]. Later that year, two other groups cloned and expressed PC synthases from *Schizosaccharomyces pombe* [12] and *Triticum aestivum* [13].

3.1.3 Direct metal binding theory

Following the cloning of the first three PC synthases, a review was published in which a mechanism for the enzyme was proposed [6]. The model was based primarily on findings from studies of a cadmium-sensitive *A. thaliana* mutant named *cad1-5*. The mutant had a mutation in the gene encoding for AtPCS1 which was predicted to result in termination of the mRNA sequence translation. The resulting translated enzyme was predicted to lack the C-terminal domain; however, *cad1-5* was still capable of synthesizing PCs. Thus, it was concluded that although the C-terminal domain is not absolutely necessary for catalysis, the generation of a mutant from this condition suggested that the C-terminal domain must have a function [12]. In the

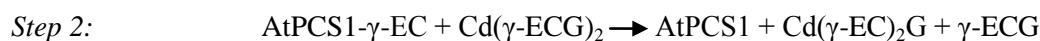
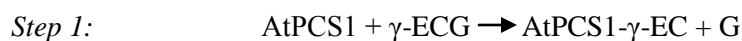
mechanism model, it was hypothesized that during enzyme activation the C-terminal domain participated in metal sensing, i.e. it would bind metals and then bring them into contact with the N-terminal domain. The N-terminal domain was designated as the catalytic center, since sequence alignments showed that this domain was highly conserved among PC synthases [6]. This theory will be referred to as the direct metal binding theory in this chapter.

The direct metal binding theory was somewhat disproved by an assessment of C-terminal domain functionality in PC synthase activation. This work focused on studies of two truncated forms of purified AtPCS1 termed PCS_Nt1 and PCS_Nt2 ending in amino acids 372 and 283, respectively. These truncated versions lacked the divergent, cysteine-rich C-terminal domain that is characteristic of PC synthases. When expressed in the cadmium-hypersensitive yeast mutant *Δyap1*, PCS_Nt1 and PCS_Nt2 were both able to confer cadmium tolerance. In addition, purified PCS_Nt1 and PCS_Nt2 displayed PC synthase activity, albeit lower than that of the full length AtPCS1. Conversely, the purified C-terminal domain of AtPCS1 (the last 202 amino acids) was not able to synthesize PCs. However, expression of this domain in *Δyap1* conferred limited cadmium tolerance to the yeast. This proved that the C-terminal domain of PC synthases was not necessary for catalysis, although its presence enhanced enzyme activity and it was effective at metal binding [9]. It should be noted however, that this data did not invalidate the direct metal binding theory completely, as activation of the enzyme via direct metal binding to the N-terminal domain was not addressed.

3.1.4 Blocked thiol theory

Shortly after the direct metal binding theory was published, a new model for the mechanism of PC synthase was reported [4], which will be referred to as the

blocked thiol theory in this chapter. The new mechanism was based on a study of metal activation and kinetics of purified AtPCS1. In this study, a computer program called SOLCON was used to predict the speciation of cadmium in PC synthase assays. In standard assay medium containing 3.3 mM GSH, 25 μ M CdCl₂ and 200 mM HEPES-BTP (pH 8), 98% of the cadmium was predicted to exist as a Cd·GS₂ complex. The concentration of free cadmium in the assay medium was calculated to be 0.1 pM [4]. These calculations were discussed along with the binding capacity and the K_L that was determined for cadmium binding to AtPCS1 by Vatamaniuk et al. [5]. Although AtPCS1 bound cadmium at high capacity, the K_L was six orders of magnitude greater than the amount of free cadmium in the assay calculated by SOLCON. Hence, it was concluded that activation of AtPCS1 via direct metal binding to the enzyme would be an unlikely scenario. A kinetic study of AtPCS1 showed that enzyme activity not only increased with increasing GSH, but also with increasing cadmium concentration [4]. This was in contrast to earlier enzyme kinetics studies, in which PC synthase was assayed in varying GSH concentrations while the cadmium concentration was held constant [2, 3]. The K_M values determined for Cd·GS₂ ($9.2 \pm 2.3 \mu$ M) and GSH (13.6 ± 3.3 mM) led the authors of this work to conclude that both GSH species act as substrates where Cd·GS₂ is considered the high affinity substrate and GSH is the low affinity substrate. The kinetic model that best described AtPCS1 catalysis was the substituted enzyme ping pong mechanism. Based upon these findings, a two-step mechanism for AtPCS1 was proposed and is described by the following reaction [4]:



It was determined that the first step of this reaction was metal independent. This was concluded from an experiment in which AtPCS1 was assayed in substrate buffer consisting of GSH containing radiolabeled glycine. In the absence of cadmium the release of free glycine was detected. When cadmium was added to the assays, the release of free glycine was increased by 100 fold and PC synthesis occurred. It was concluded that cadmium only was required for the second step of the reaction, the transfer of γ -Glu-Cys onto a GSH peptide [8].

Evidence was also reported suggesting that direct metal binding contributed to enzyme activation, but was not crucial for catalysis [4]. It was discovered that in the absence of cadmium, AtPCS1 could synthesize S-alkyl-PCs from S-alkyl-GSH: GSH molecules in which an alkyl group is bound to the side-chain sulfur. A kinetic study of AtPCS1 in which S-methylglutathione (S-methyl-GS) was used as the substrate revealed that the peptide could act as both a high and low affinity substrate in the presence or absence of cadmium. To prove that metals tightly bound to AtPCS1 did not activate the enzyme in cadmium deficient assays, AtPCS1 was incubated with ethylene glycol tetraacetic acid (EGTA), a synthetic chelator. EGTA and the metals bound to the chelator were removed via dialysis. The purified, dialyzed AtPCS1 was active in substrate buffer containing S-methyl-GS and lacking cadmium, though the addition of cadmium to the assay increased the enzyme's activity. Maximum activity was achieved at 0.5 μ M cadmium. Activity began to decrease at cadmium concentrations greater than 0.5 μ M, while inhibition was observed at concentrations greater than 5 μ M. It was concluded that the only substrate absolutely required for AtPCS1 activation was an S-substituted GSH derivative (also referred to as a blocked thiol). It was acknowledged that heavy metals can stimulate enzyme activity, but they are not necessary for catalysis [4].

The study of AtPCS1 activation by blocked thiols was continued by Romanyuk et al. [11]. In that study, a truncated version of AtPCS1 ending with amino acid 221, called AtPCStr_221, was assayed in varying concentrations of cadmium while the S-methyl-GS concentration was held constant. In these experiments, AtPCS1tr_221 was active when assayed in the absence of cadmium. However, for cadmium concentrations of about 0.1 μ M to 5 μ M, the activity of AtPCS1tr_221 immediately decreased, in contrast to full-length AtPCS1 which was initially stimulated by cadmium addition [4]. The authors concluded that in these experiments, the C-terminal domain of AtPCS1 protected the full-length enzyme from inhibition by directly binding excess cadmium ions, while AtPCS1tr_221 is susceptible to inhibition by excess cadmium ions. They also concluded that when GSH was used as a substrate the only mode of activation by cadmium was through the formation of the Cd·GS₂ which is used as the actual substrate.

In a study by Oven et al. [7], results that contradicted the blocked thiol theory were reported. In that work, purified AtPCS1 assayed in substrate buffer containing S-methyl-GS was minimally active and the addition of 500 μ M cadmium to the assay terminated enzyme activity. However, the addition of thiol-containing compounds (GSH, DTT, 2-mercaptoethanol, γ -Glu-Cys, L-cysteine, or D-cysteine) to those assays re-activated AtPCS1. In addition, unique concentrations of each thiol-containing compound were required to obtain a consistent level of activation. When reducing agents that did not contain thiols (namely ascorbic acid, sodium borohydride, imidazole, and histidine) were added to the assay, the enzyme could not be re-activated. This ruled out reduction of the enzyme as a possible mechanism for activation. Based upon these findings, the authors concluded that AtPCS1 was not activated by blocked thiols, but by thiol containing compounds that deliver cadmium to AtPCS1 resulting in enzyme activation [4].

Another group challenged the conclusion that the first step of PC synthesis by AtPCS1 (the cleavage of glycine from GSH) is metal independent [14]. In that study PC synthesis and γ -Glu-Cys formation were monitored simultaneously. These researchers reported that the formation of PCs and γ -Glu-Cys by purified AtPCS1 in the absence of cadmium were both extremely minute. Upon the addition of cadmium to the assay, the concentrations of PCs and γ -Glu-Cys increased immediately. These researchers argued that their data suggests that both of the steps necessary for PC synthase catalysis are metal dependent [14].

The data reported by Oven et al. [7] was later challenged by Rea et al. [10]. In that rebuttal, the authors stated that the high concentrations of cadmium in the assays published by Oven et al. [7] led to inhibitory thiol capping which terminated enzyme activity. They suggested that thiol-containing compounds chelated the excess cadmium in the assays which resulted in enzyme re-activation. The authors maintained that blocked thiols were the only requirement for PC synthase activation [10].

3.1.5 Direct metal binding theory revisited

Although there is strong evidence to support the theory that PC synthases are activated by blocked thiols, there remains one strong argument to suggest that direct metal binding to PC synthases is critical to enzyme function. It was reported that the addition of metal chelators to assays terminates PC synthase activity. Also, the addition of excess PCs to assays was shown to terminate *in vitro* PC synthesis by binding the very metals that activate the enzyme [15]. This finding complemented the observation that *in vitro* PC synthase activity ceases after a certain amount of time presumably by the chelation of activating metals to PCs, but activity can be restored by cadmium addition [2]. The rebuttal to this data is that chelation of metal by PCs cannot terminate enzyme activity, since the peptides themselves can be used as

substrates. It is likely that *in vivo* PC-metal complexes are transported to vacuoles which effectively depletes the substrate concentration and terminates enzyme activity [10]. It has also been shown that the addition of a synthetic chelator, ethylenediaminetetraacetic acid (EDTA), terminated PC synthase activity *in vitro* [2]. However, no data has been published that proves that enzyme activity can be recovered after the addition of EDTA, i.e. there is no proof that EDTA does not destroy the enzyme.

This chapter describes experiments designed to determine if direct metal binding to PC synthase is required for activation by testing the ability of three different synthetic chelators to terminate enzyme activity. Purified AtPCS1 pretreated with bathocuproine, a copper(I) chelator, prior to dialysis was not active in the absence of cadmium when S-methyl-GS was used as a substrate in assays. However, activity could be restored by the addition of cadmium. The activity of purified TpPCS3 also approached zero in the absence of cadmium when the enzyme was pretreated with bathocuproine prior to dialysis. Bathocuproine pretreated TpPCS3 was also stimulated by the addition of cadmium. The results suggest that bathocuproine pretreatment terminates PC synthase activity without destroying the enzyme, however, the mode of termination by this chelator could not be determined. In addition, a novel GSH-like peptide, γ -Glu-Ser-Gly, was designed to test the necessity of blocked thiols in PC synthase activation. Neither AtPCS1 nor TpPCS3 was able perform a dipeptidyl transpeptidase reaction with this peptide; however, AtPCS1 inhibition was observed when γ -Glu-Ser-Gly was added to assays in which the substrate was GSH or S-methyl-GS. The results suggest that both AtPCS1 and TpPCS3 require blocked thiols for activation.

3.2 METHODS AND MATERIALS

3.2.1 *TpPCS3 and AtPCS1 assays*

For crude cell extract assays, BL21(DE3)*plysS E. coli* crude cell extracts containing TpPCS3 and AtPCS1 were prepared for assays as described in Chapter 2. For PC₂ or hybrid pentapeptide synthesis experiments, the extracts (200 µg/mL) were assayed in 200 mM HEPES-NaOH (pH 8), 7 mM GSH and/or 7 mM S-methyl-GS where indicated. Assay samples were removed after 3 minutes as described in Chapter 2. In some experiments, longer assays were performed (up to 30 or 60 minutes) to ensure that the enzyme was not capable of new peptide formation under the stated conditions.

For purified enzyme assays in which S-methyl-GS or γ-Glu-Ser-Gly was used as the sole substrate, TpPCS3 or AtPCS1 (0.5 µg/mL) were assayed in substrate buffer containing 200 mM HEPES-NaOH (pH 8), S-methyl-GS and CdCl₂ for 15 minutes. To terminate the reaction, 5-sulfosalicylic acid was added to 5% (w/v). The samples were centrifuged for 30 minutes at 4°C, after which 300 µL of the supernatant was removed for HPLC analysis.

3.2.2 *PC₂, S-methyl-PC₂ and GSH/S-methyl-GS hybrid pentapeptide quantification*

Samples (200 µL volume) containing *S-methyl-GS* or γ-Glu-Ser-Gly were separated on a reverse-phase Alltech Adsorbosphere HS C8 3U according to Vatamaniuk et al. [4]. The elution protocol used a 3 step gradient: (1) 0.05% (v/v) phosphoric acid for 5 minutes, (2) a linear gradient achieving a final buffer composition of 0.05% (v/v) phosphoric acid and 17% (v/v) acetonitrile for 20 minutes and (3) 0.05% (v/v) phosphoric acid for 5 minutes. The flow rate was 1 mL/min. Fractions were collected in 30 second intervals by a SC100 fraction collector (Beckman). The fractions were labeled with the fluorescent amine label o-

phthaldialdehyde (OPA) and the fluorescence was read using a BioTek Synergy 4 Multi-Mode Microplate Reader. A 100 μ L volume of each fraction was added to the wells of an opaque 96-well plate. The plate reader was programmed to dispense 275 μ L of OPA solution containing sodium tetraborate (0.5 M), OPA (10 mg/mL), and 2-mercaptoethanol (5 μ L/mL) to each well. The plate was gently shaken for 2 minutes and the fluorescence emission was measured at 455 nm following excitation at 340 nm. The sensitivity of the instrument was set at 100. S-methyl-GS was used to create a standard curve (Figure 3.1). OPA labeled S-methyl-GS was stable for 15 minutes following the 2 minute mixing period (Figure 3.2). Fluorescence readings taken from the fractions assayed in plate reader were used to create a chromatogram (Figure 3.3).

For assay samples in which both GSH and S-methyl-GS or both GSH and γ -Glu-Ser-Gly were used as substrates, assay samples were separated and quantified by HPLC using pre-column derivatization with monobromobimane as described in the Methods and Materials section of Chapter 2.

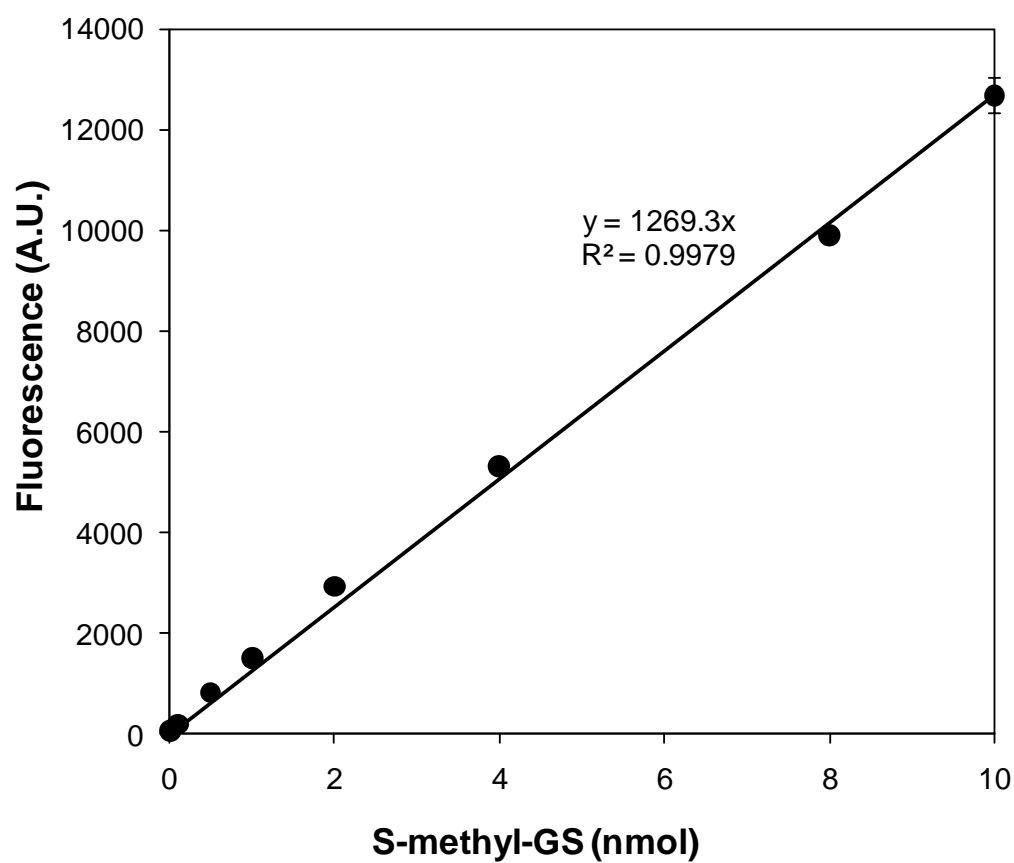


Figure 3.1. S-methyl-GS standard curve. Error bars represent means \pm S. E. ($n = 3$).

Where not shown error bars are within symbol.

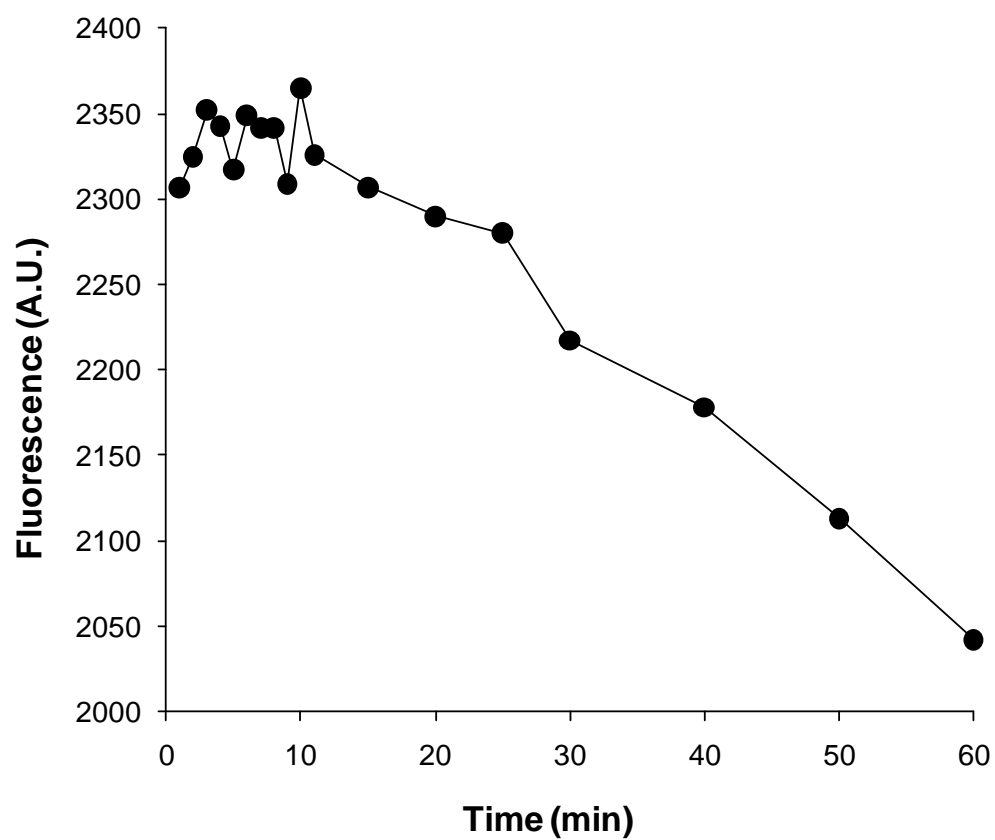


Figure 3.2. Time dependent stability of OPA labeled S-methyl-GS. S-methyl-GS was labeled as described. After the two minute mixing step, a fluorescence reading was taken at multiple time points over a period of 1 hour.

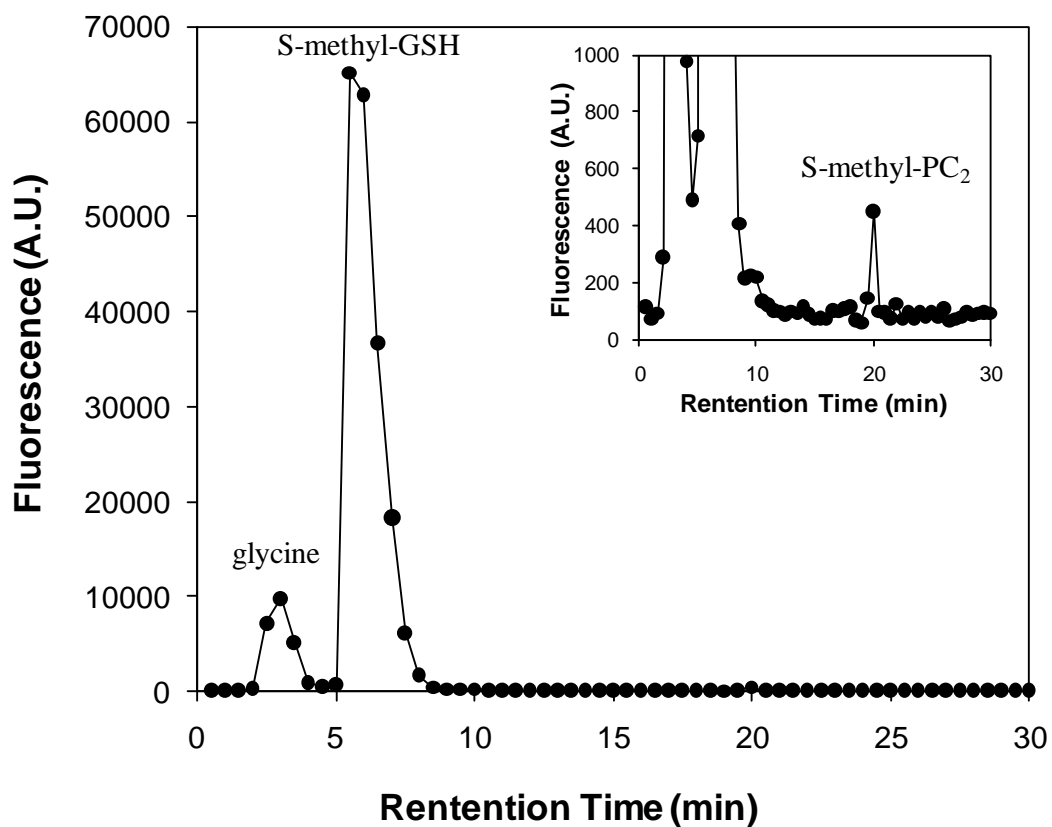


Figure 3.3. HPLC chromatogram of S-methyl-PC₂ synthesis. Purified AtPCS1 (0.5 $\mu\text{g/mL}$) was assayed in substrate buffer 3 mM S-methyl-GS for 15 minutes. Inset: Expanded view of the section of the chromatogram containing S-methyl-PC₂.

3.2.3 TpPCS3 and AtPCS1 dialysis

Purified AtPCS1 or TpPCS3 (30 µg in 200 µL volume) was incubated on ice for 1 hour in buffer containing 100 mM Tris-HCl (pH 7.4) and the indicated concentrations of bathocuproine, EGTA, EDTA or no chelator. Since the logistics of maintaining a dialysis buffer at 4°C inside an anaerobic chamber were virtually impossible, all dialysis was performed in a cold room and 5 mM TCEP was added to the pretreatment buffer of each enzyme to prevent enzyme oxidation. To remove the chelator, chelator bound metals and TCEP, the enzyme preparations were dialyzed against 300 mL of 50 mM HEPES-NaOH (pH 8). Prior to use, the dialysis HEPES buffer was made trace metal free by application to a column of Chelex Resin 100 (Bio-Rad). The resin is highly selective for divalent ions. The enzymes were dialyzed for 6 hours at 4°C in Slide-a-Lyzer mini dialysis tubes (Pierce, molecular weight cutoff of 7,000 kDa). After 6 hours, the dialysis buffer was replaced with a fresh 300 mL and the dialysis was continued for another 6 hours. Because TpPCS3 was especially sensitive to oxidation, the dialysis buffer was continually flushed with argon gas during the experiment. Centrifuge tubes, beakers and pipette tips used to perform dialyzed enzyme experiments and subsequent enzyme assays were washed once with 1 M HCL and then rinsed 3 times with Millipore ultrapure water (resistivity 18.2 MΩ·cm).

3.2.4 ICP metal quantification preparation

In preparation for ICP analysis, 6 to 10 µg of purified, dialyzed AtPCS1 were dissolved in 2 mL of 2% ultra pure nitric acid. The samples were analyzed by inductively coupled plasma mass spectrometry, ICP-MS (courtesy of Michael Rutzke, Cornell University).

3.3 RESULTS AND DISCUSSION

3.3.1 *TpPCS3 crude cell extract activity is terminated by EDTA and bathocuproine addition*

Crude cell extracts were used initially to examine the effects of various chelators on enzyme activity. *E. coli* crude cell extracts containing TpPCS3 assayed in the absence of cadmium were capable of PC₂ synthesis, indicating that there was a significant concentration of metal contamination within crude cell extracts. In an attempt to chelate contaminating metals, TpPCS3 crude cell extract was assayed in substrate buffer containing 7 mM GSH and 5 mM EDTA. The addition of EDTA to the assay reduced PC₂ synthesis but did not eliminate it. Doubling the concentration of EDTA to 10 mM further reduced enzyme activity, but again did not eliminate it (data not shown). These results led to the hypothesis that metals ions that were not chelated strongly by EDTA may activate the enzyme. Copper(I) ions were proposed as a potential candidate for activation, since copper(II) is reduced to copper(I) in the presence of reduced thiols and since EDTA predominantly binds divalent cations. However, when bathocuproine a specific copper(I) chelator, was substituted for EDTA, similar results were obtained. Interestingly, the simultaneous addition of both 5 mM EDTA and 5 mM bathocuproine abolished PC₂ synthesis (Figure 3.4).

TpPCS3 crude cell extract assayed in substrate buffer containing 7 mM S-methyl-GS and 7 mM GSH formed two new products (along with PC₂ and S-methyl-PC₂), presumably γ -Glu-Cys- γ -Glu-Cys(CH₃)-Gly and γ -Glu-Cys(CH₃)- γ -Glu-Cys-Gly (termed hybrid pentapeptides). Although the two hybrid pentapeptides overlapped at 15.3 min in HPLC chromatograms. Both peaks were distinguishable (Figure 3.5).

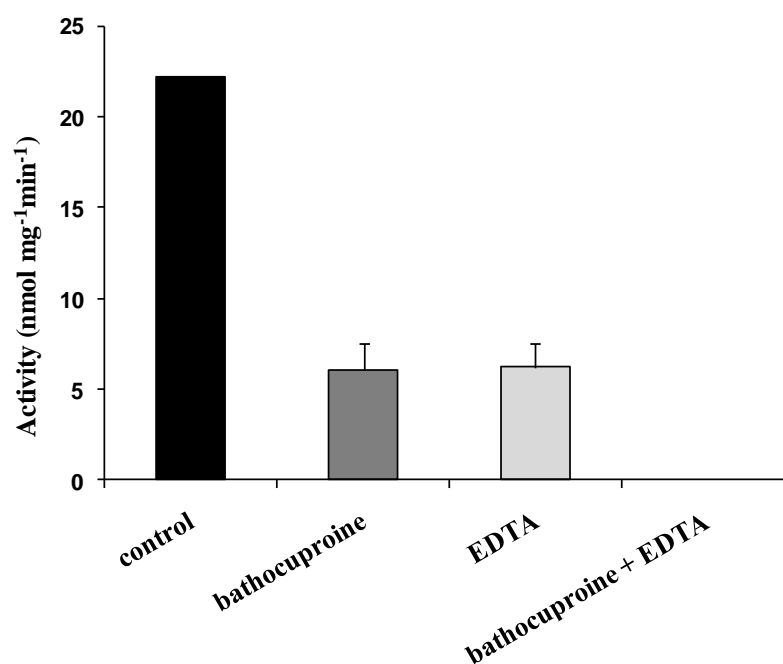


Figure 3.4. The effects of chelators on TpPCS3 activity in crude cell extract. *E. coli* crude cell extract containing TpPCS3 was assayed in substrate buffer containing 7 mM GSH and 5 mM chelator (where indicated) for 3 minutes. Error bars represent means \pm S. E. ($n = 3$).

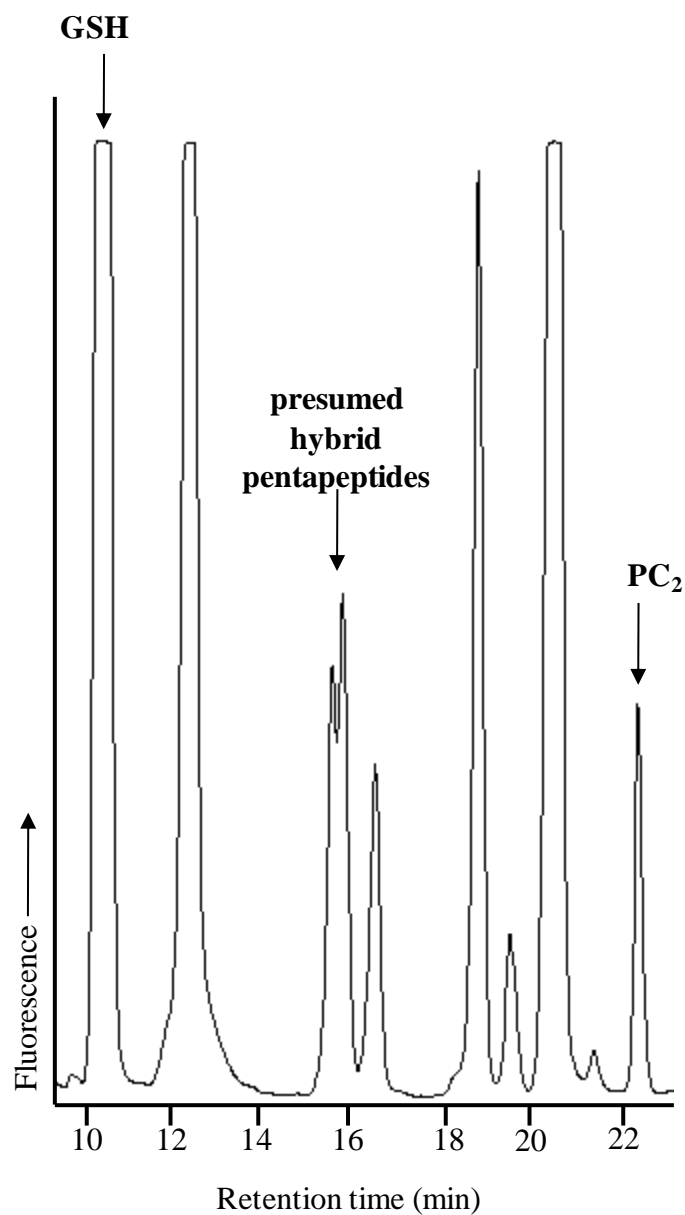


Figure 3.5. HPLC chromatogram of PC_2 and presumed hybrid pentapeptides. TpPCS3 was assayed in substrate buffer containing 7 mM GSH and 7 mM S-methyl-GS. Assay length was 10 minutes. Unlabeled peaks are derivatization reagents.

We reasoned that if AtPCS1 (and by implication other PC synthases) only requires a blocked thiol for the second step of catalysis (the transfer of γ -Glu-Cys onto another GSH molecule), and not the first (the cleaving of glycine from GSH) then the addition of a chelator directly to the assay should terminate synthesis of PC₂ and γ -Glu-Cys(CH₃)- γ -Glu-Cys-Gly but not the synthesis of γ -Glu-Cys- γ -Glu-Cys(CH₃)-Gly. However, when TpPCS3 was assayed in substrate buffer containing 7 mM GSH and 7 mM S-methyl-GS, the addition of bathocuproine and EDTA resulted in the simultaneous termination of the synthesis of all three peptides (Figure 3.6). The same result was observed for AtPCS1 when the enzyme was assayed in the same manner (data not shown). Although the results suggested that TpPCS3 requires metal to form PC₂ and the two pentapeptides, the possibility that EDTA or bathocuproine irreversibly damaged the enzyme could not be ruled out by this experiment.

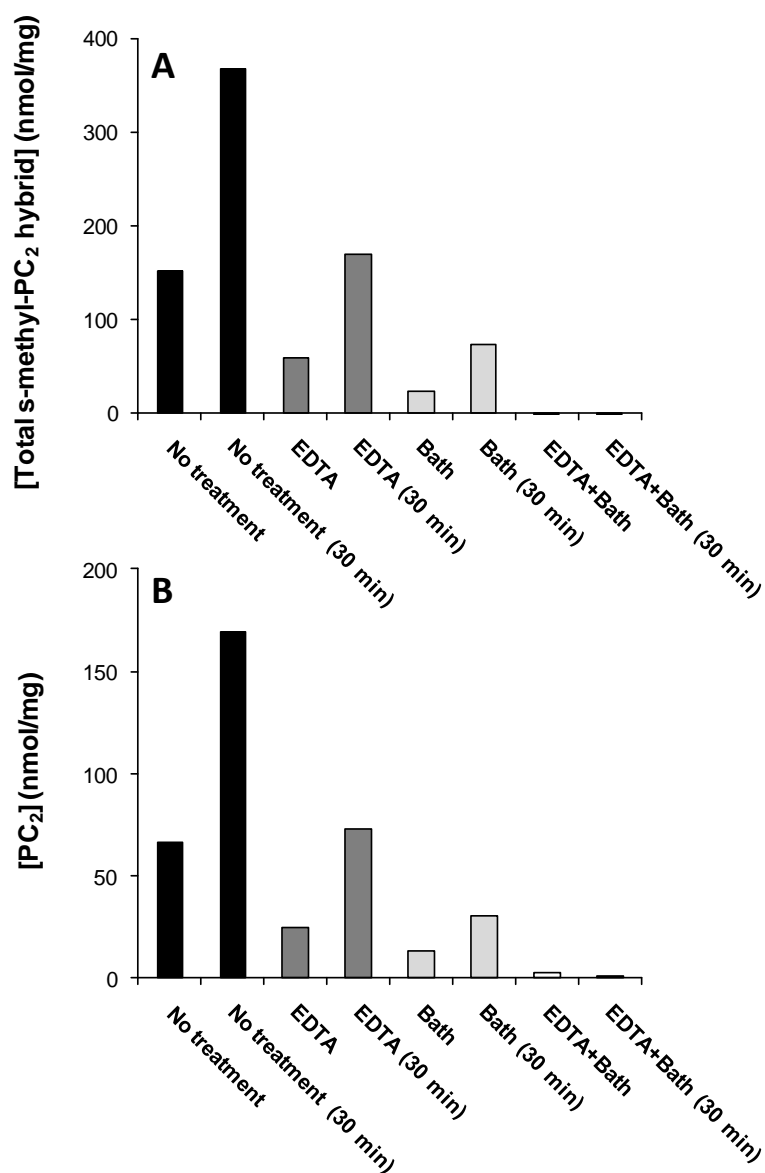


Figure 3.6. Effects of chelator addition on simultaneous TpPCS3 synthesis of PC₂ and S-methyl-PC₂ presumed hybrid pentapeptides. *E. coli* crude cell extract containing TpPCS3 (200 µg/mL) was assayed in substrate buffer containing 7 mM GSH, 7 mM S-methyl-GS and 5 mM chelator where indicated. A sample from each assay was taken at 3 and 30 minutes. The experiment performed with three different batches of crude cell extract yielded similar qualitative results. (A) Total γ -Glu-Cys- γ -Glu-Cys(CH₃)-Gly and γ -Glu-Cys(CH₃)- γ -Glu-Cys- γ -Glu-Cys-Gly and (B) PC₂.

3.3.2 AtPCS1 pretreated with bathocuproine prior to dialysis is not active in the absence of cadmium, pretreated TpPCS3 retains residual activity

As mentioned, EDTA and EGTA bind divalent cations (i.e. Cd^{2+} , Zn^{2+} or Cu^{2+}) tightly, but bind copper(I) weakly. Originally, we hypothesized that copper(I) ions in crude cell extracts were not chelated by EDTA and were available to bind to the enzyme or the substrate, GSH. Hence, bathocuproine addition was required to completely terminate enzyme activity. We also hypothesized that copper(I) ions could co-purify with PC synthases and bypass the metal contamination removal step via incubation with EGTA followed by dialysis as described by Vatamaniuk et al. [4]. In the case of AtPCS1 assayed with S-methyl-GS, the resulting contaminating copper(I) ions bound to the enzyme or a fraction of the enzyme may have created the impression that AtPCS1 does not require direct metal binding for activation. The addition of cadmium ions may have added to or replaced the copper(I) ions already bound to the enzyme, resulting in an increase in activity.

To test this hypothesis, purified AtPCS1 was incubated with 1 or 5 mM EDTA, 1 mM EGTA, 5 mM bathocuproine or no chelator (control) prior to dialysis (Figure 3.7). The enzyme (30 μg in 200 μL volume) was dialyzed against two 300 mL volumes of trace metal free HEPES-NaOH (pH 8). The final concentration of chelator in the enzyme preparations after dialysis was calculated to be 11 nM. TCEP was added to a concentration of 5 mM to preserve enzyme activity since AtPCS1 became completely oxidized during dialysis in the absence of a reductant.

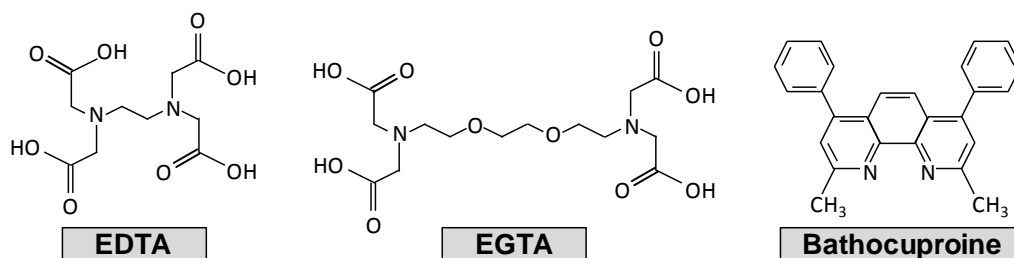


Figure 3.7. Molecular structure of metal chelators.

Following dialysis, the AtPCS1 preparations were assayed in substrate buffer containing 3 mM S-methyl-GS, with and without 0.5 μM CdCl_2 for 1 hour. No activity was measured in the instances where AtPCS1 had been incubated with either 1 mM or 5 mM EDTA. When the enzyme was pretreated with 1 mM EGTA or no chelator, activity was measured in the presence and absence of cadmium, although the addition of cadmium increased enzyme activity more than 1.5 fold (EGTA pretreatment) and 2.5 fold (no chelator). A similar result was reported by Vatamaniuk et al. [4] for EGTA-pretreated AtPCS1. Interestingly, AtPCS1 pretreated with bathocuproine prior to dialysis was not active in the absence of cadmium; however, the addition of 0.5 μM CdCl_2 restored activity to the enzyme. The same qualitative results were observed for each AtPCS1 preparation assayed in substrate buffer containing 7 mM GSH, with and without 50 μM for 1 hour. For assays in which no PC_2 formation was observed, the formation of γ -glutamylcysteine was also not detected. The data suggests that AtPCS1 requires direct metal binding to the enzyme for both steps of catalysis.

The activity of bathocuproine-pretreated and control dialyzed AtPCS1 assayed in substrate buffer containing 3 mM S-methyl-GS and varying CdCl_2 concentrations increased with increasing CdCl_2 concentration from 0 to 0.5 μM . For CdCl_2 concentrations 0.5 to 1 μM , activity of the control enzyme decreased with increasing

cadmium and activity was inhibited at concentrations higher than 1 μM . At CdCl_2 concentrations higher than 0.5 μM the activity of bathocuproine-treated enzyme was inhibited (Figure 3.8). At the cadmium concentration that elicited maximum activity a 17.4:1 molar ratio of cadmium to AtPCS1 was calculated for the assay solution.

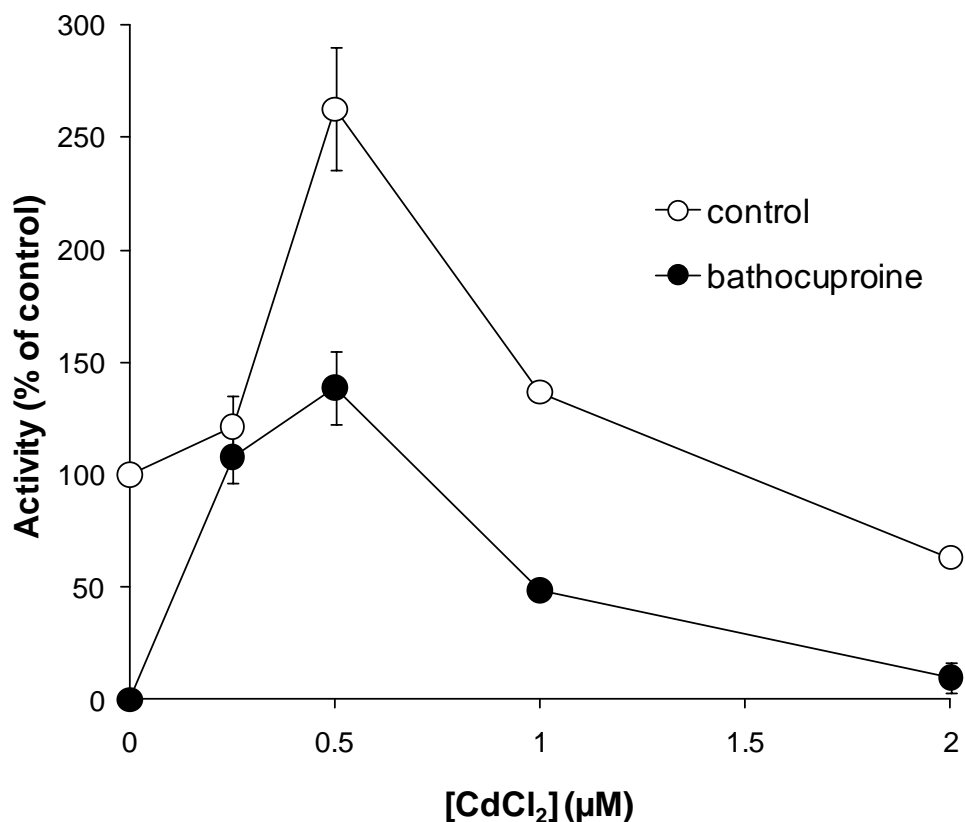


Figure 3.8. S-methyl-PC₂ synthesis by dialyzed AtPCS1. AtPCS1 (30 μg) was pretreated with 5 mM bathocuproine or no chelator (control) prior to dialysis. The dialyzed enzymes (0.5 $\mu\text{g}/\text{mL}$) were assayed in 3 mM S-methyl-GS and varying concentrations of CdCl_2 . Assay length was 15 minutes. Activities are presented as the percentage of activity measured for non-pretreated AtPCS1 assayed in the absence cadmium, which was 1.5 $\mu\text{mol mg}^{-1} \text{min}^{-1}$. Error bars represent means \pm S.E. ($n = 3$). Where not shown error bars are within symbol.

TpPCS3 pretreated with bathocuproine prior to dialysis and the non-chelator treated control were both active in the presence or absence of cadmium. Since the activity of the bathocuproine-treated enzyme was extremely low in the absence of cadmium, we hypothesized that contaminating metal ions within the substrate buffer were sufficient for activation in this case. To test this hypothesis, the HEPES component of the substrate buffer was applied to a Chelex column prior to its mixture with the other substrate buffer components. When assayed in the substrate buffer containing Chelex-treated HEPES, TpPCS3 activity approached zero, although a level of zero activity was not achieved (Figure 3.9). It is likely that TpPCS3 binds metal very tightly and that 5 mM bathocuproine, although sufficient for the removal of metal from AtPCS1, was unable to chelate all of the contaminating metals from TpPCS3. Contaminating metals within the substrate buffer may have also contributed to activation of this enzyme. Thus, bathocuproine-pretreated TpPCS3 exhibits residual activity following dialysis. From CdCl_2 concentrations 0 to $0.125\ \mu\text{M}$, the activity of bathocuproine-pretreated TpPCS3 increased with increasing cadmium concentrations. Enzyme activity decreased with increasing cadmium at concentrations above $0.125\ \mu\text{M}$ (Figure 3.9). At the cadmium concentration that elicited maximum activity a 5:1 molar ratio of cadmium to TpPCS3 was calculated for the assay solution.

The activity of the control TpPCS3 decreased immediately with increasing cadmium concentrations. We hypothesized that this was because the metal contamination in this preparation was sufficient for full activity and supplemental cadmium poisoned the enzyme.

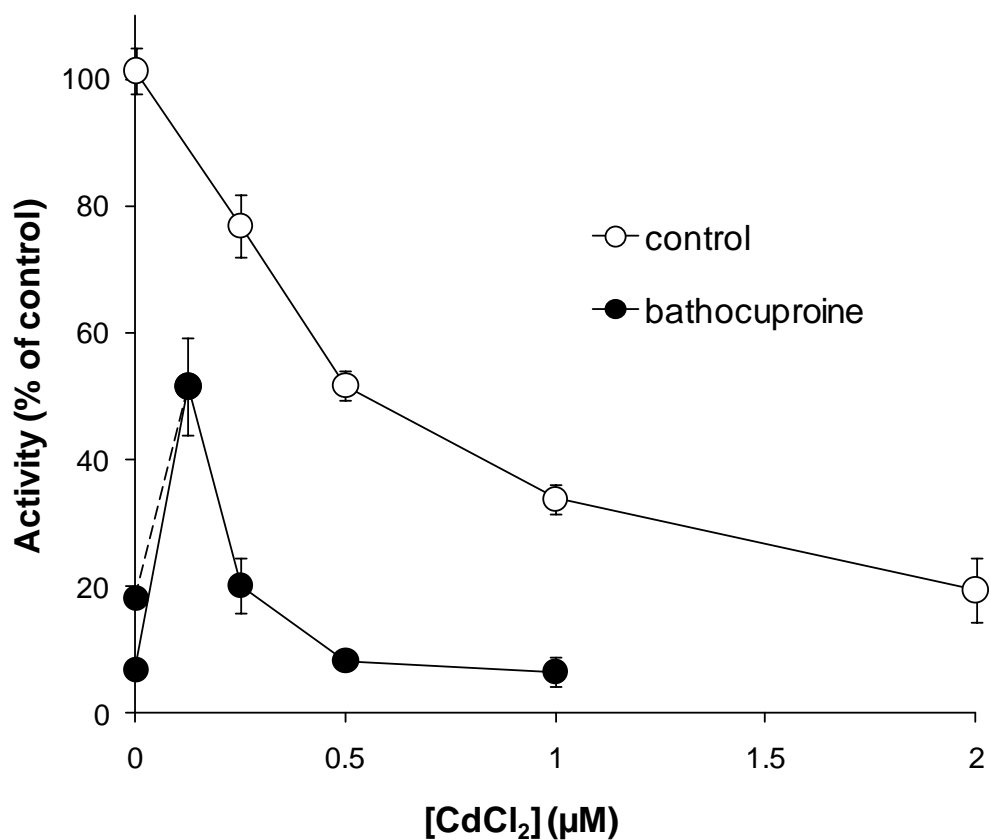


Figure 3.9. S-methyl-PC₂ synthesis by dialyzed TpPCS3. TpPCS3 (30 μg) was treated with 5 mM bathocuproine or not treated (control) prior to dialysis. The dialyzed enzymes (0.5 μg/mL) were assayed in 3 mM S-methyl-GS and varying concentrations of CdCl₂. Assay length was 15 minutes. The point at the zero cadmium concentration associated with the dotted line represents enzyme activity prior to treatment of the HEPES component of the substrate buffer with Chelex. Activities are presented as the percentage of activity measured for non-pretreated TpPCS3 assayed in the absence cadmium, which was 3.9 μmol mg⁻¹ min⁻¹. Error bars represent means ± S.E. (*n* = 3).

The relative higher sensitivity of TpPCS3 to cadmium in these assays (when compared to AtPCS1) may be explained by the number of C-terminal cysteines each enzyme contains. AtPCS1 contains 12 of these cysteines, while TpPCS3 only contains 5. As mentioned, the C-terminal cysteines of AtPCS1 appear to protect the enzyme from inhibition by free cadmium when S-methyl-GS is used as a substrate[11]. Since TpPCS3 has fewer C-terminal cysteines it may be less capable of binding added metal in these assays, which may result in stimulation and inhibition of the enzyme at lower concentrations of cadmium when compared to AtPCS1. If this is true, then the C-terminal cysteines may play an indirect role in the kinetics of these enzymes.

Interestingly, the concentration of cadmium required for the maximum activation of the bathocuproine-pretreated TpPCS3 when S-methyl-GS was used as a substrate, 0.125 μM , is strikingly close to the K_M found for $\text{Cd}\cdot\text{GS}_2$, 0.113 μM . This is in contrast to bathocuproine-pretreated AtPCS1, whose K_M for $\text{Cd}\cdot\text{GS}_2$ is four times that of the cadmium concentration that elicited its greatest activity when S-methyl-GSH was used as a substrate. Along with the finding in Chapter 2, that TpPCS3 participates in ternary complex, these data suggest that TpPCS3 requires direct metal binding for activation.

3.3.3 ICP-MS analysis of AtPCS1 dialyzed preparations does not reveal copper(I) removal by bathocuproine

An ICP-MS analysis of the dialyzed AtPCS1 preparations revealed that the cadmium, copper and zinc concentrations of the control and bathocuproine-pretreated enzymes were not significantly different. The copper and zinc concentrations in the EGTA pretreated enzyme preparation was 37% less than those measured in the control or bathocuproine-pretreated enzymes, however, its cadmium content, though low, was

significantly higher than the other two enzyme preparations (Figure 3.10). The metal contents of the enzymes determined by ICP-MS analysis do not seem to suggest that bathocuproine pretreatment is superior to the other pretreatments in terms of copper removal. Therefore, we can not conclude that contaminating copper(I) ions activated AtPCS1 when it was pretreated with EGTA. It is important to note that the concentrations of other metals that could potentially activate AtPCS1 were not evaluated in this analysis. It is possible that bathocuproine may bind another metal that is required as a structural component in the enzyme. It is also possible that these measurements are incorrect as this analysis was not performed or replicated and proper controls were not done.

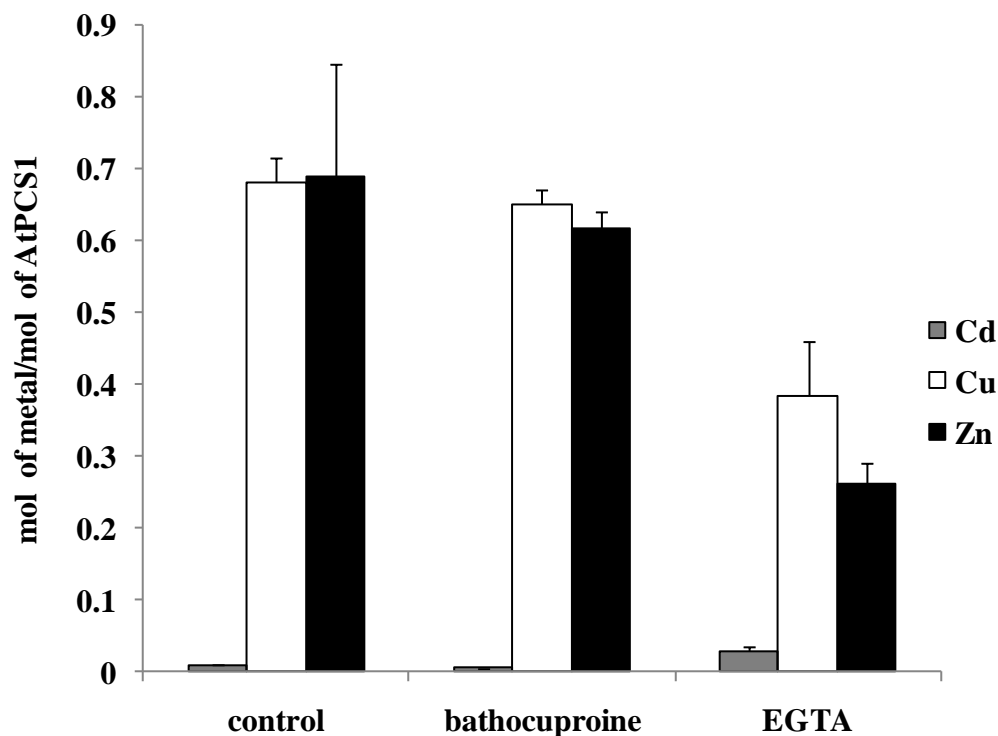


Figure 3.10. Metal content of dialyzed AtPCS1. Non treated control, bathocuproine pretreated and EGTA pretreated AtPCS1 (6-10 μ g) were analyzed by ICP-MS for cadmium, copper, and zinc concentrations.

3.3.4 EDTA-pretreated AtPCS1 activity can be restored by magnesium or calcium addition

As mentioned, AtPCS1 that was pretreated with EDTA prior to dialysis was not active in the presence or absence of cadmium. We hypothesized that pretreatment of the enzyme with EDTA may remove a magnesium cofactor from the enzyme, since EDTA has a higher affinity for magnesium than EGTA does. Interestingly, the addition of 1 mM MgCl₂ or 1 mM CaCl₂ to substrate buffer containing 50 µM CdCl₂ and 3.3 mM GSH restored activity to the enzyme. In contrast, only 1 mM CaCl₂ was capable of restoring activity to AtPCS1 when the substrate buffer contained 0.5 µM CdCl₂ and 3 mM S-methyl-GS (Table 1). The activity elicited by the addition of CaCl₂ was similar to the level of activity detected from the dialyzed AtPCS1 control assayed under the conditions of 0.5 µM CdCl₂ and 3 mM S-methyl-GS. MgCl₂ (1 mM) restored only minimal activity to the enzyme under the same conditions. We know that magnesium did not poison the enzyme in this instance, since the addition of 1 mM MgCl₂ did not alter the activity of bathocuproine-pretreated AtPCS1 assayed in the same manner. At the very least, the data shows that EDTA does not cause irreversible damage to AtPCS1. There are at least two explanations for the activation of this enzyme by calcium and magnesium. It is possible that residual EDTA was not completely removed from the enzyme preparation during dialysis and chelated the cadmium added to these assays. In this case, AtPCS1 activity may have been restored by the addition of magnesium or calcium which may bind to the residual EDTA allowing cadmium to activate the enzyme. It is also possible that since the magnesium and calcium used in these experiments was not ultrapure, contaminating metal ions capable of activating AtPCS1 were inadvertently added to the assay, which resulted in enzyme activation. In either case the discrepancy between the activation of the

enzyme by the addition of magnesium when S-methyl-GS versus GSH is used as a substrate remains unclear.

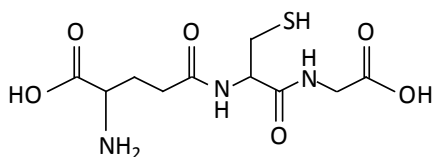
Table 3.1. The effect of CaCl_2 and MgCl_2 on EDTA-pretreated AtPCS1 activity. AtPCS1 pretreated with EDTA or bathocuproine prior to dialysis was assayed in substrate buffer containing 3 mM S-methyl-GS and 0.5 μM CdCl_2 , or 3 mM GSH and 50 μM CdCl_2 . Where indicated 1 mM MgCl_2 or 1 mM CaCl_2 was added to the assay. (+) indicates that AtPCS1 synthesized PCs or S-methyl-PCs under the listed conditions. (–) indicates that AtPCS1 was not capable of synthesizing new products. N.A. indicates that the experiment was not performed.

	GSH		S-methyl-GSH	
	MgCl_2	CaCl_2	MgCl_2	CaCl_2
EDTA	+	+	–	+
Bathocuproine	n.a.	n.a.	+	+

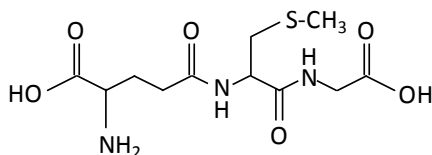
3.3.5 γ -Glu-Ser-Gly inhibits AtPCS1 activity but cannot be used as a substrate for the dipeptidyl transpeptidase reaction

It is difficult to design assays that differentiate between PC synthase activation by a blocked thiol or by direct metal binding in PC synthase activation. In assays where the enzyme is incubated with GSH and CdCl_2 , both a blocked thiol group (within $\text{Cd}\cdot\text{GS}_2$) and free cadmium ions are present. A similar environment is created in assays where the enzyme is incubated in S-methyl-GS and CdCl_2 . In an attempt to solve this problem, we designed a novel peptide that has the structure γ -Glu-Ser-Gly (Figure 3.11). This peptide has the novelty of mimicking the structure of GSH or S-methyl-GS, but does not contain a blocked thiol group or a thiol group that can bind metals. We proposed that by using this peptide as a substrate in PC synthase assays we

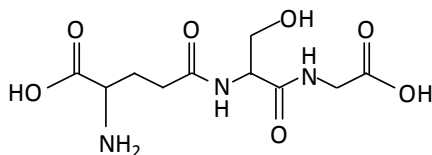
would be able to determine the necessity of direct metal binding for enzyme activation separate from the requirement for a blocked thiol, based upon the formation of a PC-like peptide, γ -Glu-Ser- γ -Glu-Ser-Gly (or lack thereof). In addition, we proposed that we would be able to determine the substrate requirements for each step of PC₂ synthesis by detecting the peptide(s) synthesized when γ -Glu-Ser-Gly and S-methyl-GS serve as potential substrates in a single experiment.



γ -glu-cys-gly
Glutathione



γ -glu-cys(SCH₃)-gly
S-methylglutathione



γ -glu-ser-gly
Novel Peptide

Figure 3.11. Molecular structures of GSH, S-methyl-GS and γ -Glu-Ser-Gly.

AtPCS1 was assayed in substrate buffer containing 3 mM γ -Glu-Ser-Gly, with and without 0.5 μ M CdCl₂. Assay samples were taken at several time points throughout the 6 hour assay. The formation of a γ -Glu-Ser- γ -Glu-Ser-Gly product could not be detected in any of the assay samples. The same result was observed for TpPCS3 assayed in 3 mM γ -Glu-Ser-Gly with and without 0.125 μ M CdCl₂. As mentioned, TpPCS3 and AtPCS1 can form mixed hybrid pentapeptides from GSH and S-methyl-GS in the form of γ -Glu-Cys- γ -Glu-Cys(CH₃)-Gly and γ -Glu-Cys(CH₃)- γ -Glu-Cys-Gly. It was proposed that TpPCS3 or AtPCS1 may be able to form similar peptides if GSH were replaced by γ -Glu-Ser-Gly. Both enzymes were assayed in substrate buffer containing 1 mM S-methyl-GS, 1 mM γ -Glu-Ser-Gly and 0.125 μ M CdCl₂ (TpPCS3) or 0.5 μ M CdCl₂ (AtPCS1). No pentapeptide formation was observed; however, inhibition of the formation of the S-methyl-PC₂ product was detected. When, AtPCS1 was assayed in substrate buffer containing 1 mM S-methyl-GS and γ -Glu-Ser-Gly (0-1 mM), S-methyl-PC₂ synthesis decreased with increasing γ -Glu-Ser-Gly concentrations (Figure 3.12). The addition of 100 μ M and 500 μ M γ -Glu-Ser-Gly inhibited S-methyl-PC₂ synthesis by 66% and 96% respectively. The γ -Glu-Ser-Gly peptide exhibited less of an inhibition effect when GSH was used as a substrate. In this case, AtPCS1 was inhibited by 12% by 0.5 mM γ -Glu-Ser-Gly and 37% by 1 mM γ -Glu-Ser-Gly. This finding is comparable to the 33% inhibition of PC₂ formation when the enzyme was assayed in 7 mM GSH and 7 mM S-methyl-GS, however, in this case the synthesis of PC₂ lost is accounted for by the formation of the two hybrid pentapeptides. The data suggests that although the novel peptide γ -Glu-Ser-Gly can interact with the active site, it cannot be used as a substrate in the dipeptidyl transpeptidase reaction.

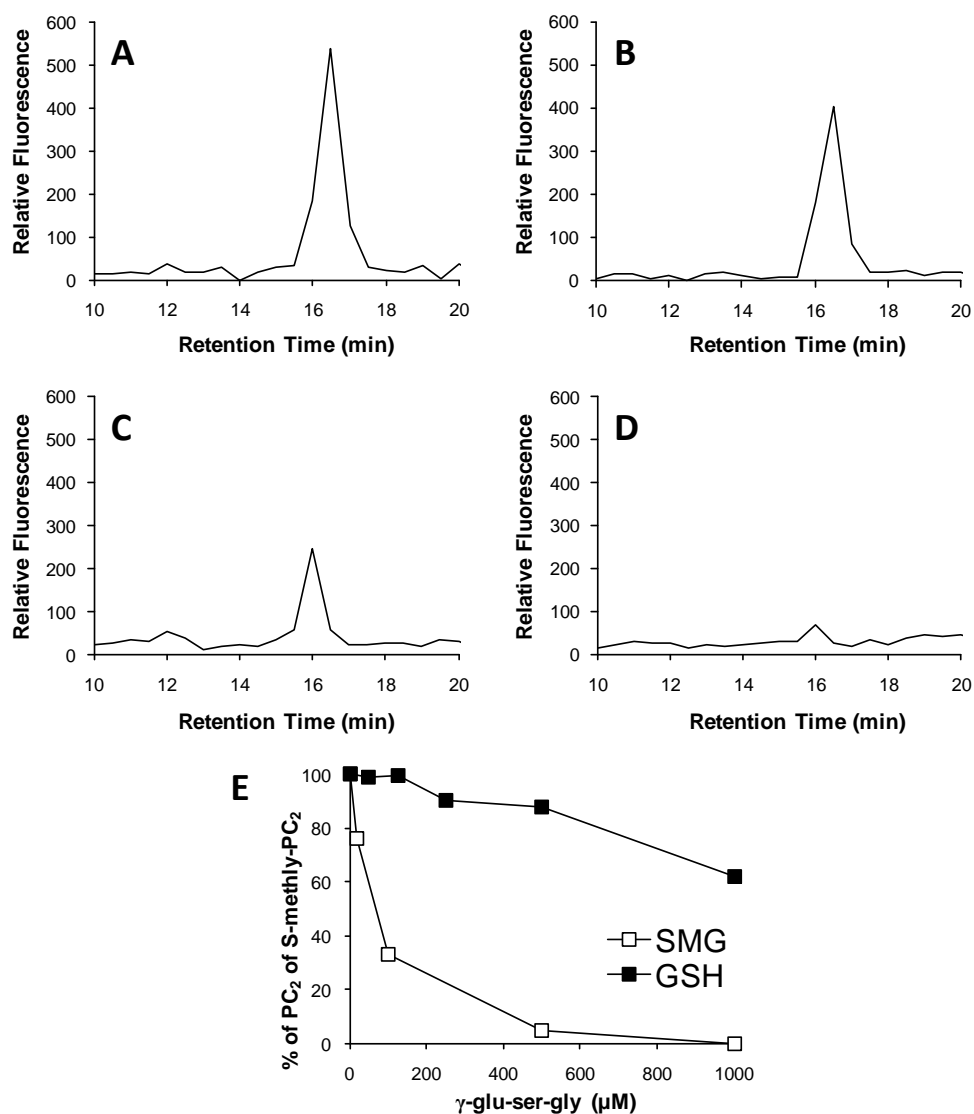


Figure 3.12. γ -Glu-Ser-Gly inhibition of PC₂ and S-methyl-PC₂ formation.

Chromatogram peaks of product formed during AtPCS1 (1 μ g/mL) assays in substrate buffer containing 1 mM S-methyl-GS, 0.5 μ M CdCl₂ and (A) No γ -Glu-Ser-Gly added, (B) 20 μ M γ -Glu-Ser-Gly, (C) 100 μ M γ -Glu-Ser-Gly and (D) 500 μ M γ -Glu-Ser-Gly. Assay length was 2 hours. (E) AtPCS1 (1 μ g/mL) was assayed in substrate buffer containing 1 mM GSH, 10 μ M CdCl₂ and γ -Glu-Ser-Gly (black squares) or 1 mM S-methyl-GS, 0.5 μ M CdCl₂ and γ -Glu-Ser-Gly (white squares). Values are the percentages of the amount of PC₂ or S-methyl-PC₂ when no γ -Glu-Ser-Gly was added.

3.4 CONCLUSION

In this chapter we demonstrated that AtPCS1 activity can be terminated and TpPCS3 activity can be significantly reduced by pretreatment with bathocuproine prior to dialysis and by using trace metal clean techniques. Bathocuproine pretreatment was more efficient at reducing AtPCS1 activity than EGTA, however, we could not prove that this is due to copper(I) binding. The finding that AtPCS1 or TpPCS3 pretreated with bathocuproine was not active (or minimally active) in the absence of cadmium when S-methyl-GS was used as a substrate, but that cadmium addition restored enzyme activity suggests that the chelator is capable of removing metal ions that are required either for enzyme activation or structure. This suggests that in some form, direct metal binding to PC synthases is essential for catalysis.

AtPCS1 was not able to use the novel peptide, γ -Glu-Ser-Gly, as a lone substrate, nor was it able to use the novel peptide to create hybrid pentapeptides when GSH or S-methyl-GS were added as substrates, however, the enzyme was inhibited by γ -Glu-Ser-Gly in the latter case. Two conclusions can be drawn from this data, either blocked thiols are required for activation of the enzyme, or the structure of the γ -Glu-Ser-Gly prevents its usage as a substrate. It is possible that although γ -Glu-Ser-Gly could fit in the active site of AtPCS1, the hydroxyl group of the serine prevented catalysis by acting as a nucleophile effectively blocking the entry of S-methyl-GS or GSH. Whether or not AtPCS1 can degrade γ -Glu-Ser-Gly to γ -Glu-Ser and free glycine could not be determined, since in the HPLC method that was used, neither product could be distinguished from the glycine and γ -Glu-Ser-Gly already present in the reaction mixture. For future studies, γ -Glu-Ala-Gly may be a better choice for the design of a novel peptide, since its methyl residue would most likely not react with the active site of the enzyme.

Notably, TpPCS3 that was pretreated with bathocuproine prior to dialysis and assayed in substrate buffer containing S-methyl-GS was activated to its maximum activity by a metal concentration similar to that of its calculated K_M for Cd·GS₂. Based upon the data from Chapter 2, and this chapter, it is conceivable that TpPCS3 could be activated by direct metal binding in a reaction where cadmium is transferred to the enzyme from a Cd·GS₂ complex and free GSH is used as a substrate.

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CHAPTER FOUR

CONCLUSION AND FUTURE RESEARCH

4.1 INTRODUCTION

In this work we successfully cloned, expressed and purified a PC synthase from *Thalassiosira pseudonana*. To our knowledge, this is first full length PC synthase to be characterized from an algal source. The characterization of TpPCS3 not only allowed us to determine specific details regarding the activation of this enzyme, but it also allowed us to make some general conclusions about all PC synthases. In this chapter, we attempt to apply these conclusions to the broader field of phytoremediation and metal homeostasis, while proposing the next steps for TpPCS3 research.

4.2 PHYTOCHELATIN SYNTHASE AS AN ENZYME FOR METAL DETOXIFICATION OR HOMEOSTASIS?

The role of phytochelatins in plants cells has been a subject of debate. Their original purpose was believed to be for metal detoxification, however, from an evolutionary standpoint the widespread distribution of an enzyme whose purpose is to detoxify heavy metals seems counterintuitive. In particular, the influx of higher concentrations of cadmium into the environment by industrial processes is too recent to play a significant role in the evolution of these enzymes [1]. Yet, cadmium is the strongest stimulator of PC synthesis. Hence, there is a growing speculation that PCs and PC synthases play a role in metal homeostasis. This idea was explored in a study of CDCA1 (a carbonic anhydrase that can use cadmium in its active site from *T. weissflogii*), in which PC₂ was used to shuttle cadmium or zinc to the enzyme *in vitro* [2]. When CDCA1 was incubated with PC₂ bound to cadmium or zinc, each metal

could be successfully and rapidly exchanged for the other. This suggested that *in vivo* phytochelatins may play a role in shuttling metals to the enzymes that need them. If this is true, this may explain why the K_M for the Cd·GS₂ substrate for TpPCS3 is so low. This results in a high rate of PC synthesis at extremely low concentrations of cadmium, which ultimately results in high intracellular PC:Cd ratios. It may be that these complexes are vital, as they are needed to shuttle metal ions (in particular cadmium) to the enzymes that need them to sustain life. However, since no biological function for cadmium has been found in plants yet, the question of why PC synthases have evolved to respond so strongly to cadmium remains unsolved.

4.3 PHYTOCHELATIN SYNTHASE TO IMPROVE PLANTS FOR PHYTOREMEDIATION

The finding that the kinetic parameters for AtPCS1 and TpPCS3 are different is not only of interest from a fundamental research perspective. Phytochelatins have been considered potential target proteins in phytoremediation research since the early 1990's [3]. Since then, attempts have been made to increase cadmium accumulation and decrease cadmium sensitivity in plants by the overexpression of PC synthase genes. These attempts have resulted in conflicting results. For instance, overexpression of AtPCS1 in *Arabidopsis thaliana* resulted in cadmium hypersensitivity [4], while overexpression of TaPCS1 in *Nicotiana glauca* resulted in increased cadmium accumulation and tolerance [5]. In a recently published article in which *CePCS1* and *AtPCS1* were both overexpressed in transgenic tobacco, it was found that the expression of the two genes had different effects on the plant [6]. This was attributed mainly to the rate of enzyme activity of each gene product. Leaf extracts containing AtPCS1 had a higher rate of PC biosynthesis than those containing CePCS1, and whole plants expressing AtPCS1 contained lower concentrations of GSH and higher

concentrations of γ -Glu-Cys than plants expressing CePCS1. It was concluded that the higher activity of AtPCS1 led to faster cleavage of GSH which disturbed plant homeostasis and led to increased sensitivity of the plants to cadmium when compared to plants expressing CePCS1. The overall conclusion was that the expression of some PC synthase genes may be more conducive to phytoremediation applications than others. The success or failure of the expression of these genes in plants may be influenced greatly by their intrinsic enzymatic rates [6]. Our data shows that large variations in the activity of PC synthases can be a consequence of their intrinsic response to metal-GS concentration. It would be interesting to see if a survey of PC synthases from various organisms could produce an ideal candidate for phytoremediation based upon studies of their enzymatic rates. Although, to create a successful phytoremediation system other enzymes and proteins involved in heavy metal detoxification pathways would need to be optimized such as proteins that participate in the transport of phytochelatins to vacuoles.

4.4 FUTURE RESEARCH

The kinetic studies [7, 8], site directed mutagenesis studies [9-11], acylation studies [10, 11], metal binding studies [8, 9], studies of enzyme domains [11, 12] and the published crystal structure of NsPCS [13] have all contributed greatly to the elucidation of the mechanism of PC synthases, however, one piece of crucial information is still needed: a crystal structure of the full length enzyme. Solving the crystal structure will serve as the ultimate proof of acylation sites, metal binding sites and perhaps the metal co-factors that are involved in the activation of this enzyme. This may prove difficult, since crystal growing and analysis is no easy task. Nonetheless, the comparison of PC synthase crystal structures from a higher plant, yeast, algae and nematode may be informative since our results suggest that PC

synthases may not be the same in terms of their active complexes. For TpPCS3 specifically, the determination of a cadmium binding constant, the presence or absence of enzyme acylation and determination of the necessity of the enzyme's C-terminal end are all additional pieces of the puzzle that can be determined without solving the crystal structure of the enzyme.

In conclusion PC synthases are complex enzymes involved in the even more complex system of cell metal detoxification and homeostasis. Although significant progress has been made in understanding these enzymes *in vitro* and *in vivo*, there are still many questions to be answered.

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APPENDIX A
CLONING AND EXPRESSION OF *THALASSIOSIRA PSEUDONANA*
PHYTOCHELATIN SYNTHASE GENE HOMOLOGUES

A.1 RATIONALE

A.1.1 Introduction

The sequencing of the *Thalassiosira pseudonana* genome revealed two additional genes other than TpPCS3 whose sequences are homologous to those of canonical PC synthases. These genes are referred to as *TpPCS1* and *TpPCS2* in this chapter. Sequence alignments of the genes with *TpPCS3* provided evidence that these genes may encode for PC synthase homologues.

A.1.2 Phytochelatinsynthase homologues

BLASTp and BLASTn searches for protein sequences that are homologous to the PC synthase (Pfam 05023) domain have revealed that several plant species have at least two potential PC synthase genes. Additionally, these searches have revealed the existence of PC synthase-like proteins having homology to the Pfam domain in bacteria [1, 2].

Arabidopsis thaliana contains a second PC synthase in addition to AtPCS1 called AtPCS2. AtPCS2 contains the catalytic cysteine, histidine and aspartic acid, as well as three of the four N-terminal domain conserved cysteines that are characteristic of PC synthases as shown in an alignment of AtPCS2 with AtPCS1 (Figure A.1) [3]. Despite its 84% amino acid sequence similarity to AtPCS1, AtPCS2 does not contribute to the synthesis of PCs in *A. thaliana*. This was observed in an *A. thaliana* mutant (*cad1-3*) that lacks *AtPCS1*, but still retains *AtPCS2*. This mutant was unable to synthesize PCs in either roots or shoots under cadmium stress and was as sensitive

to cadmium as mutants that lacked both genes. Yet, when *AtPCS2* was cloned and expressed in a PC synthase deficient *S. pombe* mutant grown in media containing cadmium, PC synthesis was detected [4]. In an attempt to explain the inability of *AtPCS2* to rescue the *cad1-3* mutant under cadmium stress, expression studies of *AtPCS2* have been performed. In one study, RT-PCR analysis of *AtPCS2* expression in *A. thaliana* suggested that its expression might be tissue specific, since *AtPCS2* expression in the roots of *A. thaliana* was significantly higher than that of *AtPCS1* [4]. In a different study, when the reporter gene *uidA*, which encodes for beta-D-glucuronidase (GUS) was expressed in *cad1-3* under the control of the native *AtPCS1* promoter (P1) or the native *AtPCS2* promoter (P2), histochemical staining of GUS and northern blotting of the gene transcripts showed that P1 was stronger than P2 in both roots and shoots. In addition, *AtPCS2* could not rescue *cad1-3* under cadmium stress when the gene was expressed under the control of P1, P2 or the strong plant promoter, CaMV. In contrast, expression of *AtPCS1* was able to rescue *cad1-3* under cadmium stress when the gene was expressed under the control of P1 or CaMV, however, *AtPCS1* could not rescue *cad1-3* when expressed under the control of P2. This suggested that the *AtPCS2* native promoter may be inherently too weak and *AtPCS2* gene transcripts may be too unstable to result in the translation of a significant amount PC synthase capable of PC synthesis *in vivo* [5].

AtPCS1	-----MAMASLYRRSLSPSPAIDFSSAEKGLIFNEALQKGTMEGFFRLISYFQTQSEPAVGGLASLSVWLNALSID-PGRKWK 77
AtPCS2	-----MSMASLYRRSL-SPPAIDFASFEGKQIFNEALQKGTMEGFFGLISYFQTQSEPAFGGLASLSMVLNLSID-PGRKWK 76
NsPCS	VIVMKLFIPVTIVIGLCLSSSQVLAQTLTLSPNLIGFNSNEGEKLLTSRSL--EDFFPLSMQFVTQVNAQVGGVASIIMVLNSLGINAPETAQY 92
AtPCS1	GPWRWFDES-----MLDCCCEPLEVVKKEKGISFGKVVCLAHCSGAKVEAFRTSQSTIDDFRKFFVKCTSENCHMISTYHRGVFKQTGTGHHFSP 166
AtPCS2	GPWRWFDES-----MLECCCEPLEIKDKGISFGKVVCLAHSSGAKVEAFRTNQSTIDDFRKYVVKCTSDNCHMISTYHQQVFKQTGTGHHFSP 165
NsPCS	SPYRVETQDNFFSNEKTKAWIAPEVVARQGMTLDELGRLASVGVKVKVNHASDINIEDFRKQVAENLKQDGNFVIVNVLKKEICQERGGHISPL 187
AtPCS1	GGYNAERDMALILIDVARFKYPPHWPFLKLLWEAMDSIDQSTGKRRGFMLISRPHREPGLLYTLSCKDESWIEIAKYLKEDVPRLVSSQHVDSVEK 261
AtPCS2	GGYNAERDMALILIDVARFKYPPHWPFLKLLWDAMDSIDQSTGRRRGFMLISRPHREPGLLYTLSCKDESWISIAKYLKEDVPRLVSSQHVDTIER 260
NsPCS	AAVNEQTDRELMVTSRYKYPPVWVKTTDLWKAMNTVDSVSQKTRGFVFSKTQD----- 242
AtPCS1	IISVVFKSLPSNFNQFIRWVAEIRITEDSNQNLSAEKSRILKQLVLKEVHETELFKHINKFLSTGVEDSLTYAAAKACQCAEILSGSPSKE 336
AtPCS2	ILYVVFKSLPANFNQFIKWMAEIRRTEDVNQNLSSEKSRILKQELLKQVQETKLFKHVDKFLSSV-YEDNLPVVAAKVYCDDEILSGYESDE 334
NsPCS	----- 354
AtPCS1	FCCRETCVKCIKGPDDSEGTVVVTGVVVRDGNQKVDLLVPSTQTECEGPEATVFAGNDVFTALLALPQTWSGIKDQALMHMKQLISMASLP 451
AtPCS2	SCCKETCVKCIKGLGEEKVTVA-----VFSGNDVFTALLALPQTWSGIKDQSLQENMKQLISMVSHP 419
NsPCS	----- 419
AtPCS1	TLLQEEVLHLRRQLQLLKRCQENKEEDDLAAPAY 485
AtPCS2	TLLQEEVLHLRRQLEMLKRCQENKEDELSAPA- 452
NsPCS	----- 452

Figure A.1. Alignment of PC synthase homologues with AtPCS1. The amino acid sequences of PC synthase homologues from *A. thaliana* (AtPCS2) and *Nostoc sp.* (NsPCS) were aligned with AtPCS1 using ClustalW2 (www.ebi.ac.uk/clustalw) and edited with Jalview. The asterisk denotes the catalytic cysteine. Catalytic histidine, aspartic acid and conserved cysteines are boxed.

As mentioned, several prokaryotic gene sequences have been identified in BLAST searches using the Pfam 05023 domain as the query sequence. The first of these genes to be cloned was a PC synthase-like gene from the cyanobacteria, *Nostoc sp.*, called *NsPCS*, [2]. The *NsPCS* amino acid sequence contains the catalytic cysteine, histidine and aspartic acid, but lacks all four of the conserved cysteines of the N-terminal domain (Figure A.1). Also, *NsPCS* is truncated in amino acid sequence length when compared to eukaryotic PC synthases. Interestingly, the termination of the *NsPCS* amino acid sequence correlates with the end of the highly conserved N-terminal domain of full length PC synthases. *In vitro* expression of *NsPCS* in PC synthase-deficient *S. cerevisiae* resulted in the depletion of intracellular GSH in conjunction with an increase in intracellular γ -Glu-Cys [2]. In another study of *NsPCS* expressed *in vitro*, the degradation of GSH to γ -Glu-Cys was accompanied by the synthesis of minute amounts of PC₂ [6]. In both studies the reactions were cadmium independent. Despite conflicting data in terms of PC synthesis, both studies concluded that the main function of *NsPCS* is the cleavage of glycine from GSH to form γ -Glu-Cys, the first catalytic step in the model proposed for AtPCS1 [7].

NsPCS is the only PC-synthase or PC-synthase homologue for which the crystal structure has been solved. Details of the crystal structure confirmed some of the key findings concluded from enzyme assays, such as the presence of the cysteine, histidine, aspartic acid catalytic triad and the formation of an enzyme-acyl intermediate [8].

A.1.3 The amino acid sequences of TpPCS1 and TpPCS2

The amino acid sequences encoded by *TpPCS1* and *TpPCS2* contain the catalytic cysteine, histidine and aspartic acid common to all PC synthases. Like *NsPCS*, both amino acid sequences are significantly shorter in length than the

sequences PC synthases as shown in an alignment of TpPCS1 and TpPCS2 with TpPCS3 (Figure A.2) [3]. Essentially TpPCS1 and TpPCS2 lack the C-terminal domain. TpPCS1 contains two of the N-terminal conserved cysteines, while TpPCS2 contains only one. The TpPCS1 and TpPCS2 proteins are only 25% and 13% identical to TpPCS3, respectively and 33% and 31% identical to NsPCS, respectively.

In this chapter, the cloning and expression of *TpPCS1* and *TpPCS2* in two different *E. coli* systems is described. This work is a continuation of the research documented by Gupton [9]. The resulting proteins were assayed in an effort to determine their functions. Though the functions of these proteins were not successfully determined, data from the assays performed provided evidence that one of the enzymes, TpPCS1 may be capable of metal binding.

A.2 METHODS AND MATERIALS

A.2.1 Vector construction, *Escherichia coli* transformation and gene expression

TpPCS1 and *TpPCS3* were cloned into ptrchis2 vectors. The resulting vectors were transformed into the *E. coli* strain TOP10F as described by Gupton [9]. pET24a(+) vectors containing *TpPCS1* and *TpPCS2* genes were constructed and transformed into BL21(DE3)pLysS *E. coli* cells in the same manner as described in Chapter 2. The FLAG tag sequence was added to the 3' end of each gene. The restriction sites NdeI and EcoRI were added to the 5' and 3' ends, respectively of *TpPCS1* and *TpPCS2* (Table 1). Both genes were expressed by following the same protocol outlined in Chapter 2.

Table A.1. PCR primers for *TpPCS1* and *TpPCS2* gene amplification. Capital letters denote restriction site sequences.

TpPCS1	
Forward:	5'-ttatCATATGggttgatcagatagc-3'
Reverse:	5'-cagaagaattgattataaagatgatgatgataaataataaGAATTCaaat-3'
TpPCS2	
Forward:	5'-ttatCATATGaccttggtggaatcc-3'
Reverse:	5'-ctaaaaaagaaggcggattataaagatgatgatgataaataataaGAATTCaaat-3'

A.2.2 Western blot analysis

Western blotting was performed as described in Chapter 2.

A.2.3 *Escherichia coli* cadmium sensitivity assays

To determine if the expression of *TpPCS1* and *TpPCS3* in TOP10F cells could rescue *E. coli* under cadmium stress, overnight cultures of *E. coli* expressing *TpPCS1*, *TpPCS3*, or empty ptrchis2 vector were used to inoculate 200 μ L of LB-AMP

containing CdCl₂ and 1 mM IPTG in a 96 well plate. To determine if the expression of *TpPCS1*, *TpPCS2* or *TpPCS3* in BL21(DE3)plysS cells could rescue *E. coli* under cadmium stress, overnight cultures of *E. coli* expressing *TpPCS1*, *TpPCS2*, *TpPCS3* or empty pET24a(+) vector were used to inoculate 200 µL of LB-KAN-CAM containing CdCl₂ and 0.6 mM IPTG in a 96 well plate. For both *E. coli* strains, the cultures were diluted to OD₆₀₀ = 0.1 prior to induction. The cultures were incubated at room temperature and the OD₆₀₀ was measured every hour.

A.2.4 Crude cell extract assays and HPLC analysis

BL21(DE3)plysS *E. coli* crude cell extracts containing *TpPCS1* and *TpPCS2* were prepared for assays as described in Chapter 2. For PC₂ synthesis experiments, the extracts (200 µg/mL) were assayed in 7 mM GSH, 5 mM TCEP and 200 mM HEPES, with and without 50 µM CdCl₂. For GSH degradation assays the cell extracts (200 µg/mL) were assayed in 500 µM GSH, 200 mM-HEPES and 5 mM TCEP. Samples were taken every 30 minutes for 2 hours for both PC₂ synthesis and GSH degradation assays. Both PC₂ and GSH degradation assays were performed at varying pH from 5 to 10. Potassium hydroxide was used to alter the pH. Assay samples were derivatized with mBBR and analyzed by HPLC as described in Chapter 2.

A.3 RESULTS AND DISCUSSION

A.3.1 TpPCS1 and TpPCS2 expression

TpPCS1 and *TpPCS2* were previously cloned into the ptrchis2-TOP10Fsystem by Gupton [9]. As in the transformation of the *TpPCS3*-ptrchis2 vector construct into TOP10F cells as described in Chapter 2, positive transformants of the *TpPCS2*-ptrchis2 vector construct could not be obtained without the addition of supplemental GSH to the transformation plates. Although *TpPCS2* was successfully cloned and

transformed into the system, expression of the gene did not yield a protein that could be detected by western blot [9]. The transformation of the *TpPCS1*-ptrchis2 vector into TOP10F cells yielded many positive transformants containing the *TpPCS1* gene in the correct orientation, however, expression of *TpPCS1* yielded low concentrations of the gene product.

To increase the concentration of proteins encoded by both genes, *TpPCS1* and *TpPCS2* were cloned and transformed in the pET24a(+)-BL21 system. At least ninety percent of the colonies resulting from the transformation of both vector-gene constructs contained the gene of interest. *TpPCS1* and *TpPCS2* were both successfully expressed in BL21(DE3)pLysS cells. The resulting proteins migrated as bands of size 44 kDa and 41 kDa for TpPCS1 and TpPCS2, respectively. The sizes of both proteins were in agreement with their predicted molecular weights plus the size of the FLAG tag.

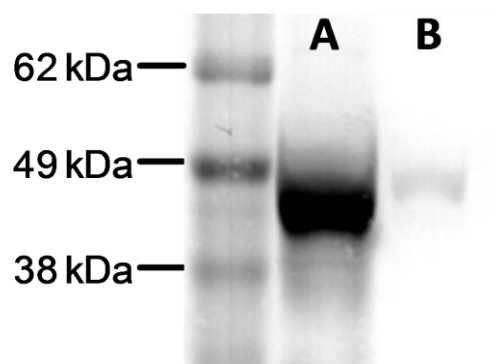


Figure A.3. Immunoaffinity detection of TpPCS1 and TpPCS2 in soluble *E. coli* extracts. *E. coli* cell extract containing (A) TpPCS2 and (B) TpPCS1 (20 µg total protein) were subjected to PAGE and western blot analysis. The blot was probed with an anti-FLAG antibody. Sizes of a molecular weight standard run in parallel are indicated.

A.3.2 Expression of TpPCS1 confers cadmium resistance

TpPCS1, TpPCS2 and TpPCS3 were expressed in two *E. coli* strains: TOP10F and BL21(DE3)plysS. TOP10F cells expressing *TpPCS1* were as resistant to cadmium stress as cells expressing *TpPCS3*. Both were more resistant than expression of the empty vector control in TOP10F cells under the same conditions (Figure A.4). However, PC₂ was not detected in the crude cell extract of cells expressing *TpPCS1*, nor was it detected when crude cell extract containing TpPCS1 was assayed in substrate buffer containing GSH with and without cadmium. The expression of *TpPCS2* was not included in this experiment since as discussed, no protein could be detected from its expression.

In contrast, the BL21 *E. coli* strain could not be used for cadmium sensitivity testing since there was no difference in the growth of the BL21 cells transformed with the empty pET24a(+) vector, *TpPCS1*, *TpPCS2* or *TpPCS3* when grown in the same cadmium concentrations as shown in Figure A.4. In this case, each transformant displayed the same resistance and sensitivity to cadmium stress regardless of the vector-gene construct that it contained, suggesting that BL21 cells are inherently more resistant to cadmium, than TOP10F cells. No PC₂ formation or intracellular GSH degradation was detected during expression of *TpPCS1* or *TpPCS2* in this strain or in crude cell extract assays (Data not shown).

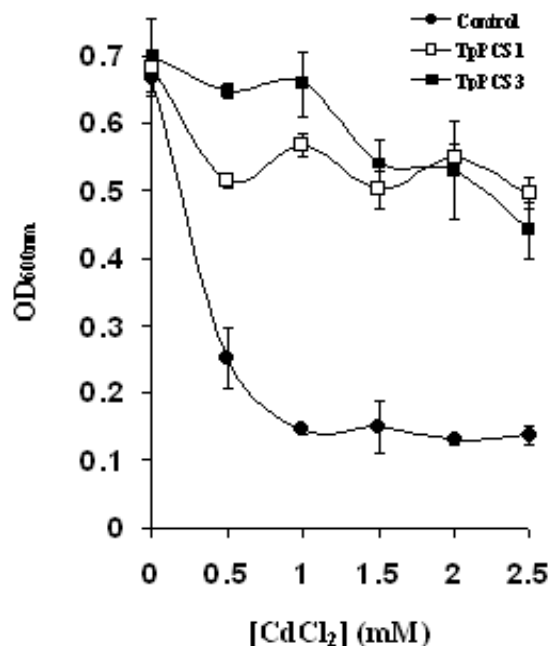


Figure A.4. Cadmium tolerance of TOP10F *E. coli* expressing *TpPCS1* and *TpPCS3* in *ptrchis2*. Overnight cultures of *E. coli* expressing *TpPCS1*, *TpPCS3* or the control (empty *ptrchis2* vector) were used to inoculate 200 μ L of LB-AMP containing CdCl₂ and 1 mM IPTG in a 96 well plate. The inhibition curves here represent 9 hours of incubation. Error bars represent means \pm S. D. ($n = 3$).

A.4 CONCLUSION

The increased cadmium resistance conferred to *E. coli* by expression of *TpPCS1* is strange, since it is not coupled with the synthesis of PC₂. As discussed in Chapter 3, the C-terminal domain of AtPCS1 expressed by itself in *E. coli* does confer some cadmium resistance. Notably, the TpPCS1 protein contains 10 cysteine and 11 histidine residues, which may significantly contribute to the metal binding potential of the enzyme. However, it is impossible to determine the contribution of these residues to metal binding by amino acid sequence alone. The reason for the lack of activity for TpPCS1 and TpPCS2 is unclear. It is likely that these proteins have some function,

since, like TpPCS3 their genes are constitutively transcribed in *T. pseudonana* [10]. It is possible that the genes require post-translational modifications that are not performed by *E. coli*. This is true of some members of the cysteine protease family such as papain, which is translated as a prepropeptide, called prepropapain. The pre and pro domains must be cleaved from the protein before it can be activated. It is also possible the TpPCS2 protein was rendered inactive by mutations that were potentially introduced during the cloning of its gene. The translation of the cloned gene sequence to an amino acid sequence revealed that 4 of the amino acids were different from what predicted. None of these amino acids mutations took place at the predicted active site, however their presence may caused a change in structure that terminated enzyme activity. The TpPCS1 protein did not contain any mutations. Expression of *TpPCS1* and *TpPCS2* in a cadmium sensitive eukaryotic system may be a better choice in an effort to determine the function(s) of these proteins.

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